Delayed Thymocyte Development Induced by Augmented Expression of p56^{lck}

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Summary

Accumulating evidence supports the contention that CD4 and CD8 receptor molecules play a critical signaling role during thymocyte development. The lymphocyte-specific protein tyrosine kinase (p56^{lck}), by virtue of its physical association with these surface components, provides a likely candidate for the biochemical signal transducing element required for these effects. To investigate the function of p56^{lck} in T lymphocytes, transgenic mice were produced that carry either the wild-type *lck* gene or a mutated *lck* gene encoding a constitutively activated form of p56^{lck} (p56^{lckF505}). Both transgenes were expressed in thymocytes under the control of the *lck* proximal promoter element. A large set of founder animals was obtained in which steady-state accumulation of *lck* transgene mRNA directly correlated with transgene copy number, suggesting that this transgene contains a dominant control region. Progeny of these founders exhibited a transgene-dependent dose-related decrease in the production of thymocytes bearing functional antigen receptors. This effect was strictly dependent on p56^{lck} activity, in that both wild-type and mutated versions of the genes induced similar effects with differing efficiencies. Remarkably, even a twofold increase in p56^{lck} abundance was sufficient to substantially disrupt the appearance of functional thymocytes. These results indicate that thymocyte maturation is regulated in part by signals derived from p56^{lck}.

Production of mature T lymphocytes requires the proliferation of immature thymic precursors, selection of cells bearing particular specificities of the clonotypic heterodimeric antigen receptor (TCR), and subsequent export of functionally mature cells from the thymus to peripheral lymphoid organs (1). Signals generated through the TCR complex influence the survival of T cell progenitors in the thymus (2), and the activation state of mature peripheral T lymphocytes (3). The TCR is composed in most cases of α and β chains in physical association with five additional transmembrane proteins: the γ, δ, and ϵ proteins of the CD3 complex, and the η and ζ chains (4–6). It is widely believed, by analogy with other receptor systems, that the signaling pathway from the TCR involves activation of a phoshoinositide (PI)[1]-specific phospholipase C, leading to production of diacylglycerol and inositol trisphophate, which in turn provoke activation of protein kinase C and the release of Ca^{2+} from intracellular stores, respectively (7). In support of this model, resting T lymphocytes can in general be fully activated to lymphokine secretion by treatment with a combination of calcium ionophore (e.g., ionomycin) and phorbol esters (e.g., PMA; reference 8).

Nevertheless, recent studies indicate that lymphokine gene expression can in some cases be achieved in the absence of PI hydrolysis or protein kinase C activation (9, 10). Such reports have encouraged a search for additional signaling mechanisms that might couple the TCR/CD3 complex to metabolic changes within the cell. Of particular interest has been the observation that engagement of the TCR/CD3 complex produces rapid changes in cellular protein phosphorylation (11). For example, both antigen and mitogen stimulation of T cell lines provoke tyrosine phosphorylation of the CD3 ζ chain (11, 12), and even more rapid tyrosine phosphorylation of other substrates (13, 14). Tyrosine phosphorylation of CD3 ζ has also been documented in immature thymocytes undergoing selective processes in the thymus (15). Since protein tyrosine kinases are implicated in the control of cell growth (16), these findings suggest that the TCR/CD3 complex may directly regulate the activity of a protein tyrosine kinase during T cell activation.

[1] *Abbreviations used in this paper:* hGH, human growth hormone gene; PI, phosphoinositide.

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Among the candidate enzymes potentially capable of mediating tyrosine phosphorylation after lymphocyte stimulation, the product of the *lck* gene is particularly attractive. A member of the *src* gene family, the *lck* gene encodes a lymphocyte-specific membrane-associated protein tyrosine kinase ($p56^{lck}$) that was originally identified by virtue of its overexpression in a murine lymphoma cell line (17, 18). Recent studies have shown that $p56^{lck}$ is physically associated with both CD4 and CD8 molecules that assist in antigen recognition by T cells (19–23). An increasingly persuasive data set favors the view that some fraction of CD4 or CD8 molecules (and presumably their associated $p56^{lck}$) interacts with the TCR-CD3 complex during antigenic stimulation (24, 25). In addition, antibody-mediated crosslinking of CD4 can in some cases be shown to directly activate $p56^{lck}$ and to augment phosphorylation of the CD3 ζ chain (26). These observations have led to the suggestion that the CD4 and CD8 polypeptides function by positioning $p56^{lck}$ within the antigen recognition complex such that its catalytic activity can be modulated by receptor occupancy (27).

To investigate the role of $p56^{lck}$ in T cell signaling, we have generated transgenic animals in which $p56^{lck}$ levels were systematically augmented. Previous studies have shown that the activity of $p56^{lck}$ is itself regulated by phosphorylation of a COOH-terminal tyrosine residue (Tyr505), and that substitution of phenylalanine for tyrosine at this position increases its ability to catalyze the accumulation of cellular proteins containing phosphotyrosine (28, 29). With this information in mind, *lck* genomic constructs were used to direct the expression of wild-type $p56^{lck}$ and $p56^{lckF505}$ in transgenic mice. Here we report that animals expressing the *lck*F505 transgene, or high levels of the wild-type *lck*Y505 transgene, exhibit disturbances in thymopoiesis. The nature of these alterations suggests that the level of endogenous $p56^{lck}$ is a critical factor in regulating thymocyte development.

Materials and Methods

Transgene Construction. The 3′ coding region of the *lck* gene was reconstructed using an XmnI fragment of the *lck*F505 cDNA in the pNUT vector (30) that contained 625 bp of the human growth hormone gene (hGH) 3′ untranslated region sequence encoding a consensus polyadenylation site. This fragment was ligated to a 1.8-kb HindIII fragment containing exons 9–11 of the murine *lck* gene, and the remainder of the gene was reconstructed using previously described genomic clones (31).

Production of Transgenic Mice. Isolated F_2 zygotes of C57BL6/J × DBA/2 mice were injected with a solution of 2 ng/μl purified linear DNA in TE buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA). After 24 h, viable embryos were transferred into the oviducts of recipient pseudo-pregnant females.

Preparation and Quantitation of Transgenic Genomic DNA. Integration of transgenic constructs into the mouse germline was assessed by the presence of hGH sequences in mouse tail DNA. Briefly, nucleic acids obtained after proteinase K digestion of tail explants were extracted with phenol/chloroform and precipitated with ethanol. Approximately 500 ng of recovered DNA was denatured, spotted onto nitrocellulose, baked, and hybridized with labeled probe. The probe consisted of sequences complementary to the 3′ untranslated region of hGH (32) labeled with ^{32}P by random priming (33). To permit quantitation of integrated sequences, known quantities of the transgene were resuspended in salmon sperm DNA and used as standards in quantitative densitometry.

Detection and Quantitation of Transgene RNA in Mouse Tissues. RNA was recovered from homogenized tissue or single cell suspensions using the guanidine-isothiocyanate method and fractionated through CsCl as described (17). RNA blotting experiments were performed as previously described (17). Quantitation of transgene mRNA was performed both by solution hybridization (34) using an hGH-specific oligonucleotide probe that was labeled with ^{32}P (30), and by densitometric analysis of RNA slot blots after hybridization using the hGH probe (isolated and labeled as above). Quantities of RNA loaded for densitometry were verified by hybridization of probes specific for the 3′ untranslated region of *lck*, or for elongation factor 1α, which served as a reference standard. TCR β chain transcripts were detected by hybridization with a human Cβ probe (35).

Antibody Staining and Flow Cytometric Analysis. Single cell suspensions obtained from lymphoid organs were depleted of erythrocytes using ammonium chloride lysis (36). Leukocytes recovered were stained for surface expression of CD4, CD8, and CD3 surface molecules, as previously described (37), using biotinylated GK1.5 (38), FITC-labeled 53-6.71 (Becton Dickinson & Co., Mountain View, CA), and biotinylated 500AA2 (39), respectively. Detection of biotinylated antibodies was facilitated using PE-conjugated streptavidin (Caltag Labs, San Francisco, CA) as a second step reagent. Multiparameter flow cytometric analysis was carried out on FACSCAN®, or FACSTAR®, and FACSTAR-PLUS® cell sorters (Becton Dickinson & Co.). Each analysis included 10,000 events collected in list mode files and analyzed using FACSTAR® Consort 30 software.

Immunoblotting. Whole cell lysates of thymocytes were boiled in SDS-PAGE loading buffer and analyzed by 12% SDS-PAGE analysis. After electrophoresis, proteins were transferred to nitrocellulose, blotted, and developed as described (17) using rabbit antiphosphotyrosine antiserum (the kind gift of Dr. Chris Wilson, University of Washington, Seattle, WA) as the primary reagent.

Results

Transgenic Constructs. Transcription of the murine *lck* gene is regulated by two widely separated promoter elements (31, 40, 41). The proximal (or downstream) promoter element is active during all stages of thymocyte development (42; our unpublished data), and sequences including this element have been used to direct expression of heterologous genes in the thymocytes of transgenic mice (37, 43). To augment $p56^{lck}$ levels in thymocytes in vivo, we assembled the constructs shown in Fig. 1 *A*. The pLGY and pLGF transgenes shown in Fig. 1 *A* consist primarily of *lck* genomic sequences, beginning 1.0 kb 5′ of the *lck* transcription start site through the coding region of the murine *lck* gene up to and including exon 11. The remainder of the *lck* coding sequence is contributed by murine *lck* cDNA sequences containing either tyrosine (Y505) or phenylalanine (F505) codons at position 505 (28). The transgene transcript is terminated at a single polyadenylation site provided by the 3′ untranslated region segment of the hGH gene (32). The presence of hGH sequences in the transgene constructs permits unambiguous

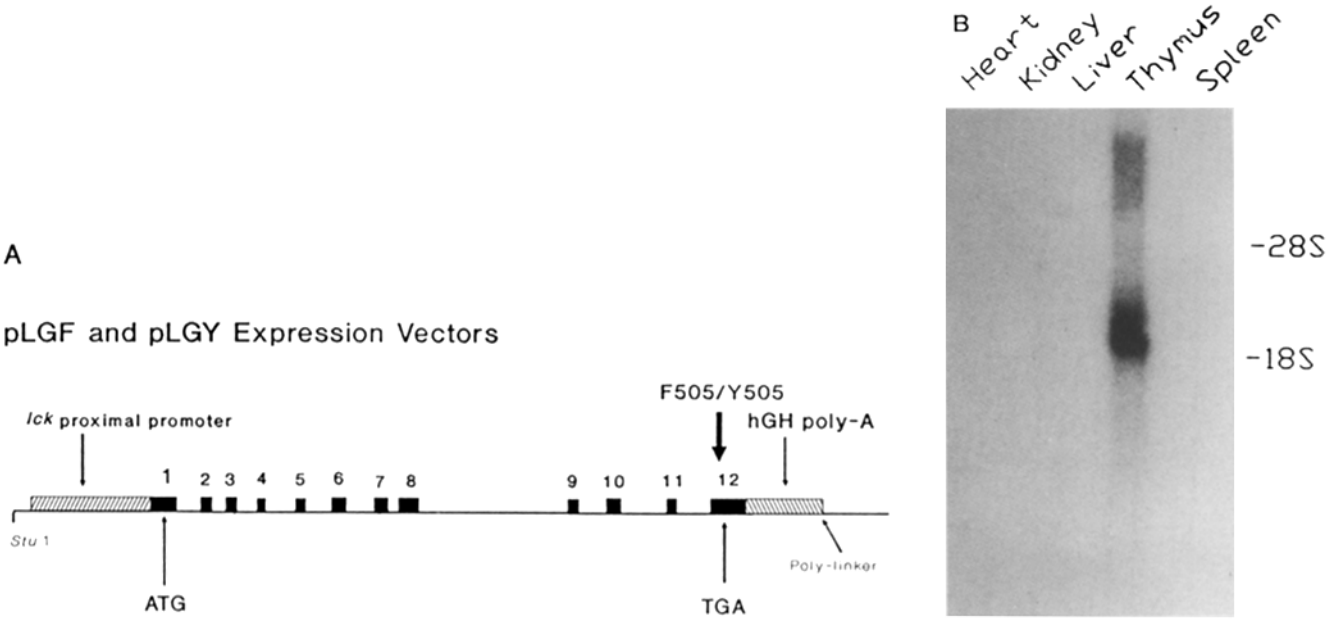


Figure 1. Diagrammatic representation of the pLGF and pLGY transgene constructs and in vivo expression pattern. (*A*) pLGF and pLGY constructs contain 11.2 kb of murine genomic *lck* sequence, including 1.0 kb 5′ to the proximal transcription start site (31) and exons 1–12 of the *lck* structural gene. A portion of exon 12 sequence was obtained from the murine *lck* cDNA encoding either the wild-type gene with tyrosine at position 505 in the pLGY construct, or a point mutation replacing phenylalanine for tyrosine at 505 in pLGF. The polyadenylation signal for these constructs is provided by 625 bp of 3′ sequence from the hGH gene. (*B*) 10 μg of total RNA recovered from indicated tissues of pLGF transgenic animal no. 671 was subjected to electrophoresis through formaldehyde-containing agarose, blotted, and hybridized with a probe specific for 3′ hGH sequences. Migration positions of eukaryotic ribosomal RNAs are indicated to the right of the figure.

detection of transgene integration in tail DNA and transgene expression in cellular RNA.

A total of 14 independent pLGF founders and 10 pLGY founders were obtained by microinjection of (C57BL/6 × DBA/2)F_2 embryos. The characteristics of these animals (and progeny obtained by crossing these animals with C57BL/6J mice) are described below.

pLGF Expression Correlates with Transgene Copy Number. Transgene-encoded transcripts accumulate to high levels only in the thymocytes of pLGF mice (Fig. 1 *B*). Not surprisingly, independent pLGF founders differed dramatically with respect to their expression levels of transgene-derived mRNA. Fig. 2 demonstrates that these differences in transgene expression correlate reasonably well with the number of pLGF transgene copies integrated. The level of endogenous *lck* mRNA in thymocytes is ~12 pg/μg total mRNA (8). Thus, our

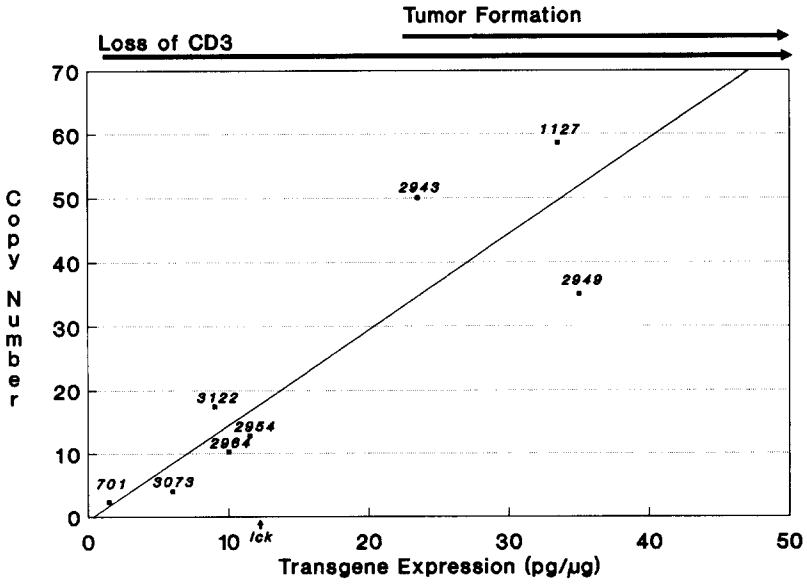
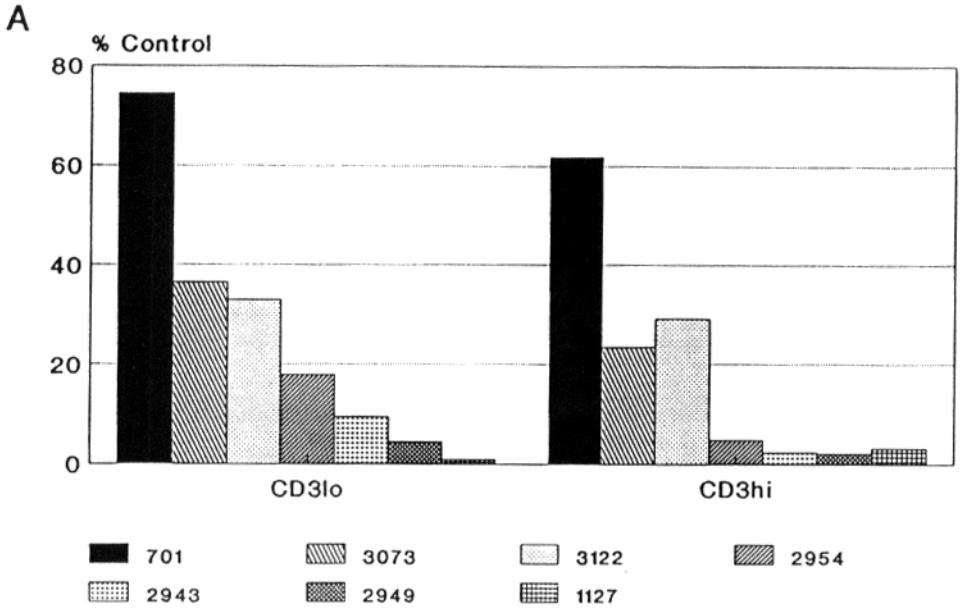


Figure 2. pLGF transgene expression is correlated with copy number and thymic phenotype. Transgene mRNA levels were determined either by solution hybridization assays or by quantitative densitometry. The number of transgene copies integrated into tail DNA was similarly assessed by quantitative densitometry using isolated transgene sequences as standards. Lines above the figure indicate the characteristic phenotypes observed over each dose interval. The level of endogenous *lck* mRNA is indicated at the bottom of the figure.



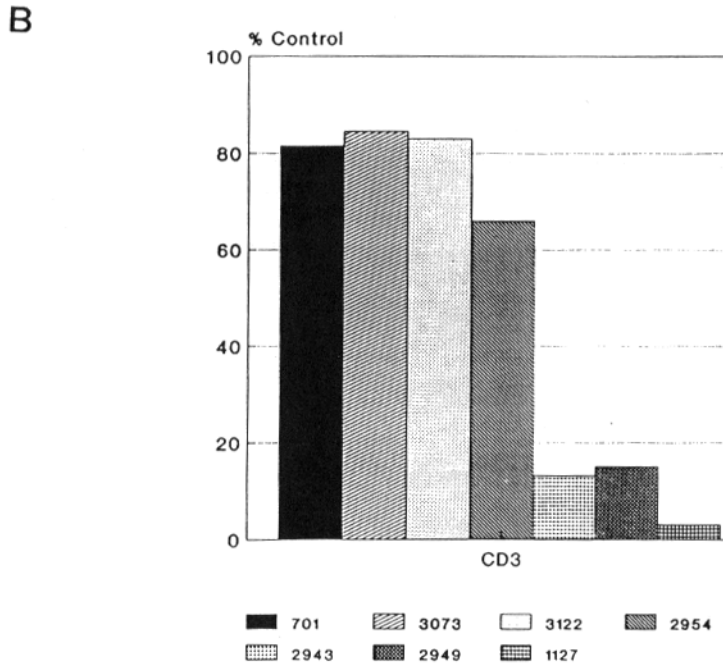


Figure 3. Representation of CD3$^+$ cells is dependent on the quantity of pLGF transgene expressed. (*A*) Thymocytes from pLGF transgenics and matched littermate controls were analyzed by quantitative flow cytometry for the percentage of cells expressing CD3. Values are expressed as the number of CD3$^+$ cells in the thymus of transgenics as a percentage of the number of CD3$^+$ cells in the littermate control. (*B*) A similar analysis on peripheral lymph node of independent pLGF founders. For both panels, animals are arranged in order of expression level from lowest (701) to highest (1127).

transgene expression levels, which range from 1.4 pg transgene mRNA/μg total RNA in the lowest expressing founder line (701; two to three transgene copies) to as high as 33.8 pg transgene mRNA/μg total RNA in the 35 copy-containing 1127 founder line (Fig. 3), result in an incremental increase in *lck* mRNA abundance in these lines of between 10 and 300%. Historically, in most transgenic animals generated using a variety of transcriptional regulatory elements, expression levels vary idiosyncratically and do not correlate with transgene copy number (44). The pLGF transgene may therefore contain a thymocyte-specific dominant control region, analogous to those described in the β-globin and CD2 loci (45, 46). From a practical standpoint, this feature of proximal *lck* promoter activity proved useful: the phenotype of pLGF

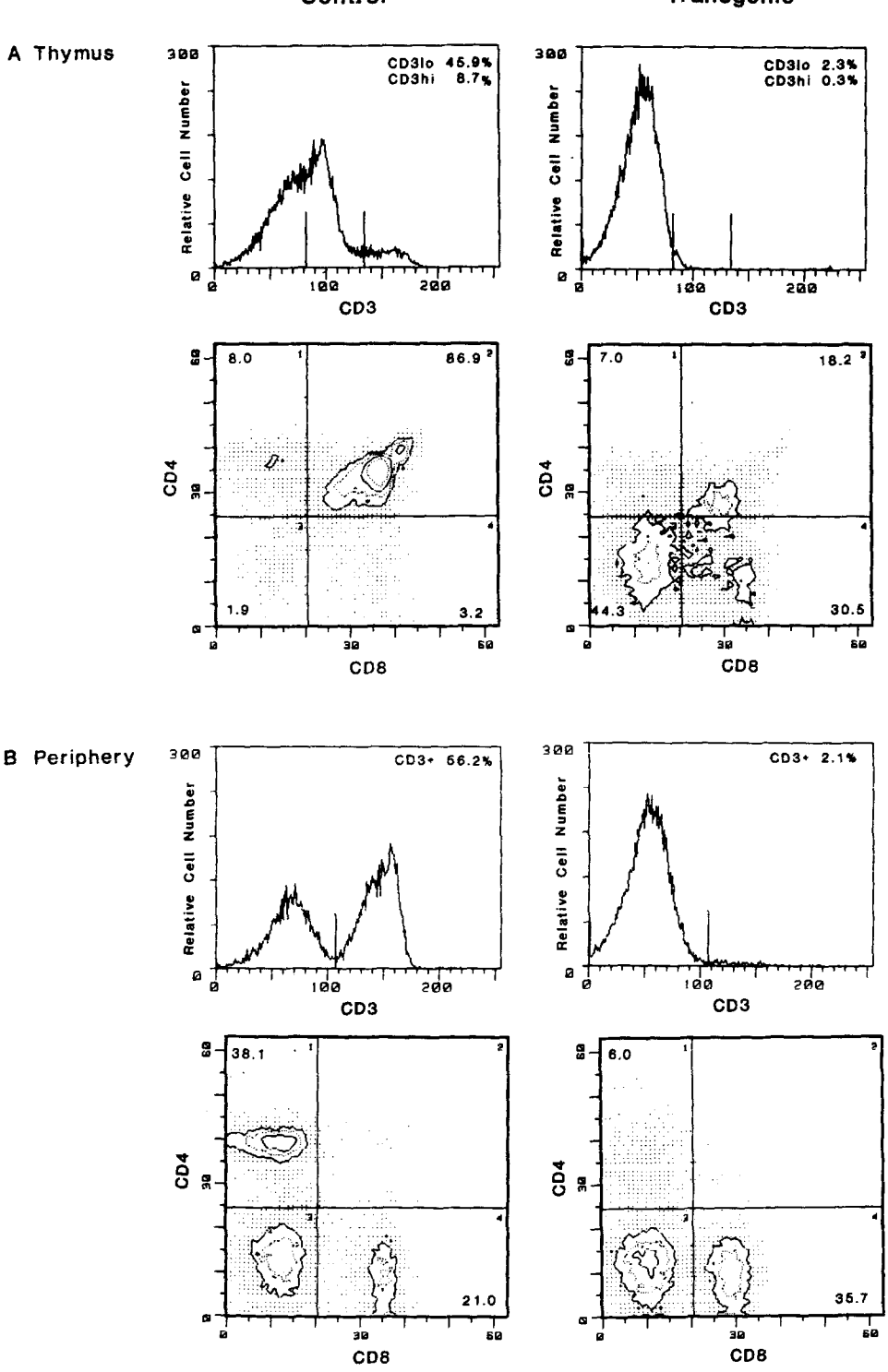


Figure 4. Maturational abnormalities in the thymic and peripheral lymphoid cell compartments of pLGF mice. Fluorescence staining profiles of surface CD3, CD4, and CD8 molecules obtained using normal littermate or pLGF transgenic (1127 line) thymocytes (*A*) or peripheral lymph node cells (*B*).

animals could be reliably predicted by simple assessment of transgene representation in tail DNA (see below).

Dose-related Variation in the Surface Phenotypes of pLGF and pLGY Animals. All transgenic animals expressing pLGF mRNA exhibit disturbances in thymocyte maturation. In a previous report (47), we described the development of thymic tumors in those mice that express high levels of the pLGF and pLGY transgenes. Although this effect is dramatic, the predominant consequence of increased $p56^{lck}$ expression in developing thymocytes is delayed or arrested maturation. This maturational arrest does not dramatically affect thymus cellularity, as the average number of thymocytes recovered from

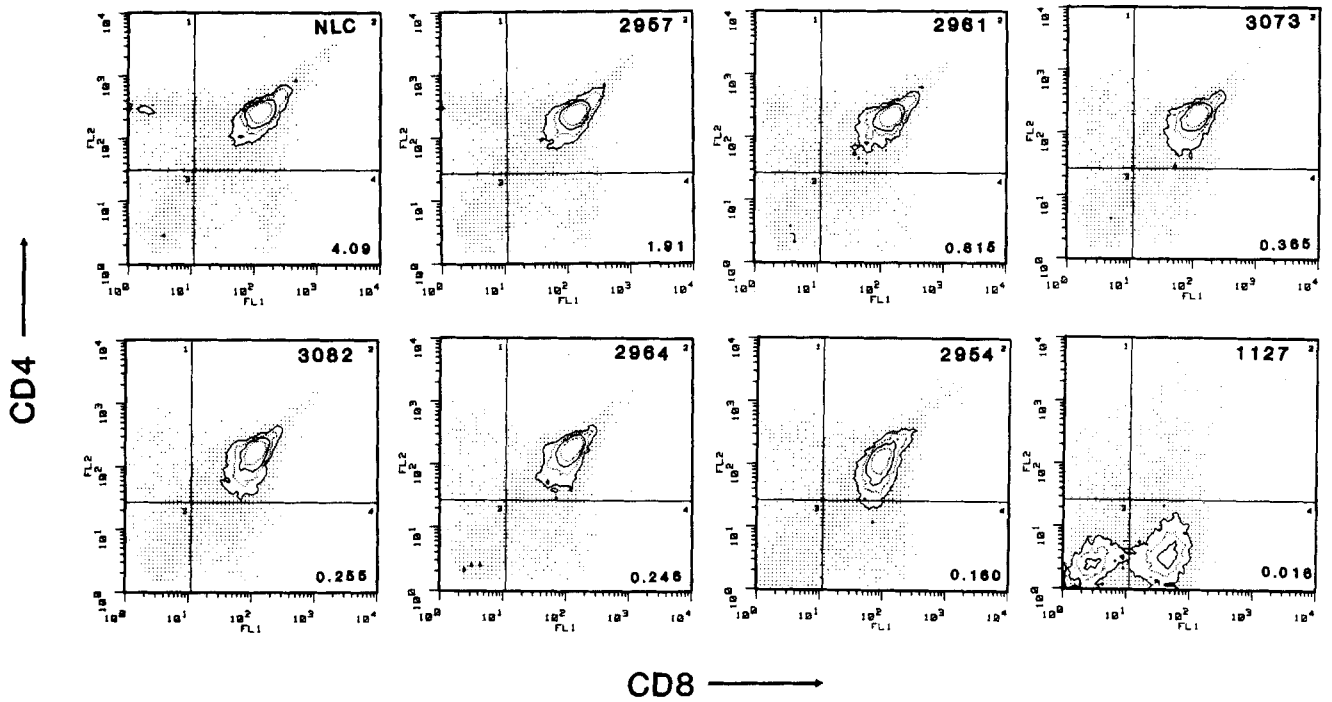
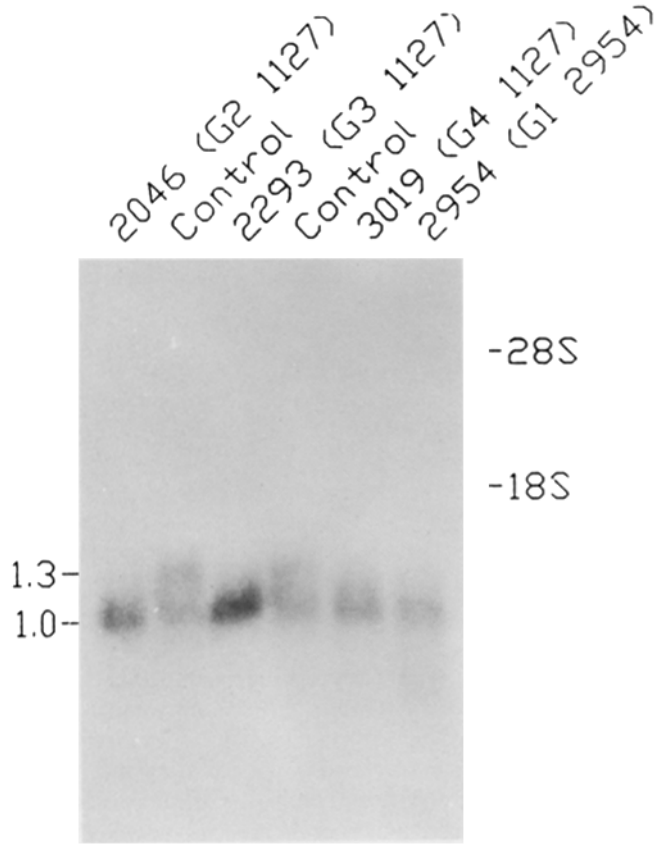


Figure 5. Correlation between extent of maturational disturbance and levels of *lck* transgene expression in thymocytes from pLGF mice. Thymocytes recovered from indicated animals at 8 wk of age were analyzed by dual parameter flow cytometry for the expression of CD8 (*x*-axis) and CD4 (*y* axis), as previously shown. CD4 to CD8 ratios of single-positive thymocytes are indicated in the lower right quadrant in each panel. Animals are arranged in order of expression level from lowest (2957 to highest (1127).



individuals of each pLGF and pLGY line at ~4 wk of age are similar to age-matched littermate controls (2.0×10^8 in the transgenics as compared with 1.7×10^8 for controls). However, many fewer of these Thy-1$^+$ thymocytes are CD3$^+$ when compared with littermate controls. Analysis of 12 independent pLGF lines revealed that the ability to produce CD3$^+$ thymocytes correlated inversely with pLGF expression level. Thus, as steady-state levels of pLGF transcripts increase, the representation of CD3$^+$ mature thymocytes declines (Table 1 and Fig. 3 *A*).

Transgenic animals that express pLGF at double to triple endogenous levels (~20–30 pg/μg total RNA; including the 2943, 2949, and 1127 lines) manifest the most severe phenotypic disturbances. Fig. 4 *A* documents typical findings in the thymus of a 3.5-wk-old animal representative of this group (1127 line). Although >98% of the thymocytes in these animals are Thy-1$^+$ (data not shown), CD3$^+$ cells are virtually absent. This striking decrease in thymic CD3 expression is accompanied by a dramatic alteration in CD4 and CD8

Figure 6. Reduced levels of full-length TCR β chain transcripts in pLGF thymocytes. 10 μg of total RNA recovered from thymocytes of indicated animals was analyzed by northern blotting using a probe specific for the constant region of the TCR β chain. Migration positions of eukaryotic ribosomal RNAs are indicated at the right of the figure. Migration positions of alternate forms of β chain transcripts are indicated in kilobases at the left of the figure.

Table 1. *Phenotype Associated with p56^{lck} Overexpression*

Phenotype	Founder	n*	Transgene expression‡	Copy number§	Tumor formation‖	CD3 (%C)	
						Thymus	Periphery¶
			pg/μg				
pLGFhi	1127	23	33.8	58.6	+	1.0	3.0
	2949	7	35.0	25.0	+	4.0	15.0
	2943	1	23.4	50.0	+	9.5	13.1
	671	1	ND	ND	ND	11.0	16.0
pLGFmid	2954	13	11.7	12.8	–	13.0	66.0
	2964	5	10.0	10.3	–	12.0	42.4
	3082	7	ND	ND	–	10.6	51.4
	3122	1	9.0	17.4	–	33.0	83.0
	3073	9	6.0	4.0	–	36.5	84.5
	629	1	ND	ND	ND	53.0	92.9
pLGFlo	2961	3	ND	3.7	–	62.0	85.0
	701	13	1.4	2.3	–	70.3	81.6
	2957	5	ND	1.2	–	76.5	100.0
	795	6	ND	1.7	–	78.8	107.2
pLGYhi	4220	12	75.0	49.2	+	11.9	48.5
	7233	5	ND	64.5	+	35.9	35.6
pLGYmid	1610	1	11.0	ND	ND	68.6	93.1
	7240	1	ND	28.1	–	88.1	117.0
	1627	1	ND	ND	ND	98.6	85.7
pLGYlo	1570	1	1.5	ND	ND	99.2	99.5
	7246	1	ND	12.3	–	123.9	97.6
	1592	4	ND	ND	–	92.2	99.4
	1572	3	ND	ND	–	101.3	101.7

* Number of individual animals analyzed in each founder line.
‡ Transgene mRNA expression in pg/μg total cellular RNA.
§ Transgene integrants/genome equivalent.
‖ Tumor formation by 8 wk of age.
¶ Total number of CD3$^+$ cells in thymus or spleen as a percentage of normal littermate control value.

staining profiles, with relative enrichments of >20-fold in double-negative cells (from 1.9% to 44.3%) and 10-fold in CD4$^-$8$^+$ cells (to >30%). Double-positive and CD4$^+$ cells are concomitantly reduced in number. In addition, virtually all of the CD8$^+$ cells that remain exhibit an immature CD8^{lo} phenotype, expressing approximately fivefold less surface CD8 than normal thymocytes. Fig. 5 provides histograms documenting the spectrum of CD4 and CD8 expression observed in representative pLGF animals of similar ages arranged according to the level of transgene expression. Clearly, as *lck* transgene expression increases, the relative proportion of cells with mature CD4$^+$8$^-$ or CD4$^-$8$^+$ phenotypes declines, and a dramatic decrease in the CD4/CD8 ratio among such single-positive thymocytes becomes evident.

Another measure of the accumulation of immature cells in the thymuses of pLGF animals was obtained by examining the representation of transcripts derived from the TCR β chain locus. Rearrangement of β chain gene segments occurs relatively early in thymocyte development. Transcripts (1.0 kb) derived from D$_\beta$-J$_\beta$ joining events are ordinarily observable by day 14 of fetal life, and complete V$_\beta$-D$_\beta$-J$_\beta$-containing transcripts (1.3 kb) become predominant by fetal day 18 (48). In thymocytes from adult pLGF mice, however, the vast majority of β-containing transcripts derive from incompletely rearranged D$_\beta$-J$_\beta$ joining events (Fig. 6). Hence, with respect to cell surface phenotypes and TCR gene rearrangements, overexpression of p56^{lck} retards the normal developmental sequence.

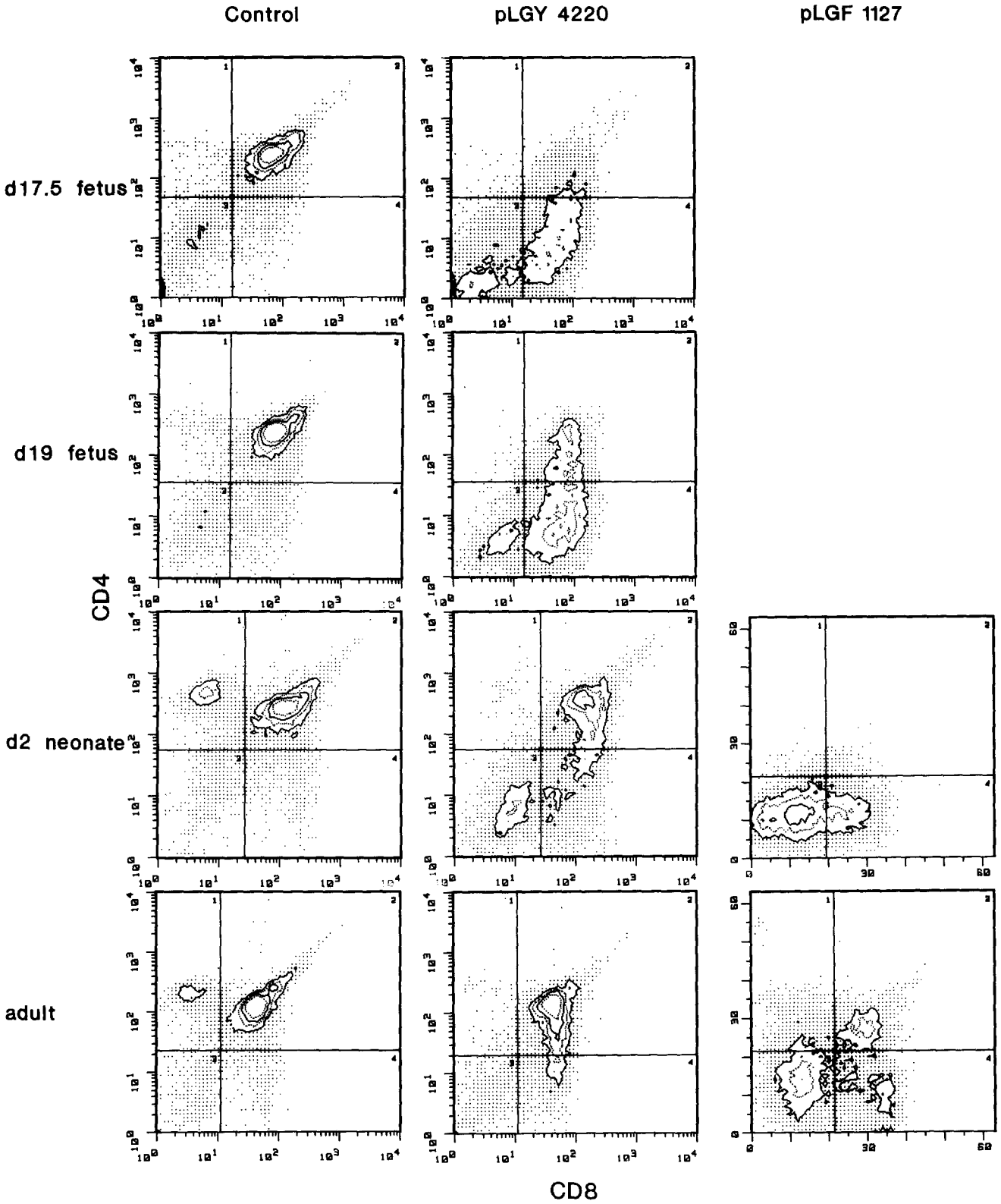


Figure 7. Developmentally delayed acquisition of surface CD4 and CD8 in transgenic thymocytes. Shown are fluorescence profiles of surface CD4 and CD8 molecules on thymocytes of either control, pLGY, or pLGF transgenic mice at the indicated stages of life.

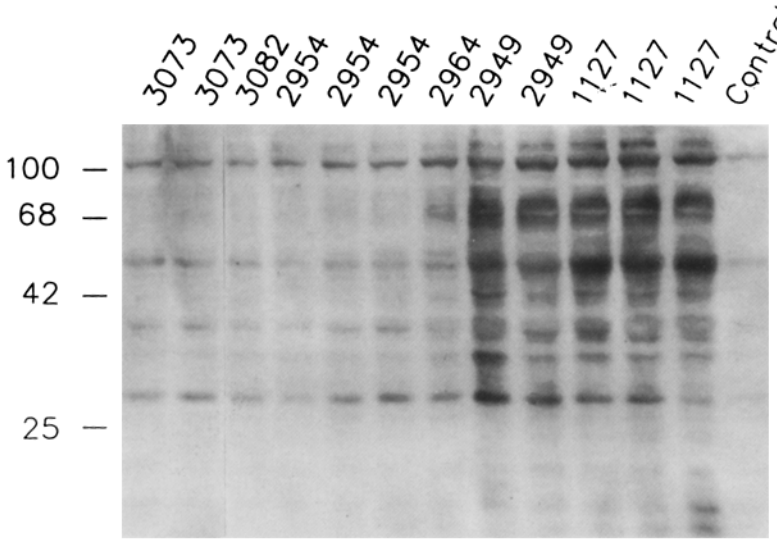


Figure 8. Increased phosphotyrosine levels in pLGF transgenic thymocytes. Whole cell lysates (50 μg total protein) obtained from dispersed thymocytes of animals from indicated pLGF transgenic lines and a control animal (Control) were analyzed by immunoblotting using a phosphotyrosine-specific antiserum (obtained from Dr. Chris Wilson, University of Washington). Transgenic samples are arranged with increasing transgene expression levels from left to right. The migration positions of protein standards in kilodaltons are indicated to the left of the figure.

Not surprisingly, the accumulation of T cells in peripheral lymphoid organs was also impaired by expression of the *lck*F505 transgene (Table 1; Figs. 3 *B* and 4 *B*). Accordingly, in most cases it was possible to rapidly assess the degree of disruption of thymopoiesis by enumeration of $CD3^+$ cells in peripheral blood (data not shown). Again, decreased representation of mature $CD3^+$ lymphocytes correlated inversely with pLGF transcript abundance.

Overexpression of the Normal lck Gene Is Not Benign. To determine whether the alternations in thymopoiesis observed in pLGF mice were uniquely provoked by the *lck*F505 mutant transgene, we examined animals bearing a wild-type *lck* construct (pLGY). Animals with transgene expression at approximately endogenous levels (e.g., no. 1610, with 11 pg pLGY mRNA/μg total RNA) exhibited a modest reduction in CD3-expressing thymocytes (Table 1). The phenotype observed in animals expressing pLGY at this level thus approximates that observed in pLGF mice expressing sevenfold lower levels of transgene transcripts, providing an in vivo estimate for the extent of activation of $p56^{lck}$ induced by the F505 mutation. Indeed, animals overexpressing the pLGY transgene at extraordinarily high levels (at least twofold higher than the highest expressing pLGF line) also develop thymic tumors (Table 1, and reference 47). Thus, augmentation of total $p56^{lck}$ activity, whether by simple overexpression or by expression of the *lck*F505 mutant, profoundly disrupts thymocyte maturation.

Thymocyte Maturation in pLGF and pLGY Transgenic Animals Proceeds with Delayed Kinetics. To examine the basis of the *lck* transgene effects, we studied thymocyte ontogeny in high-expressing pLGF and pLGY mice. Fig. 7 illustrates the CD4 and CD8 staining profiles of pLGF and pLGY thymocytes (1127 and 4220 lines, respectively) during early life. Both of the *lck* transgenic animals appear to generate thymocytes with normal surface receptor expression, but do so with greatly delayed kinetics. Thus, the CD4/CD8 expression profile of an adult 4220 pLGY animal resembles that seen in a normal animal at 19 d of gestation. Although the appearance of $CD3^+$ cells in pLGY mice might represent successful maturation of those few thymocytes in which transgene expression is for some reason decreased, it is remarkable that the extent of disruption of development correlates extremely well with the level of *lck* transgene expression (Table 1 and data not shown). This result suggests that thymocyte maturation can only proceed successfully when *lck* gene expression (or more precisely, $p56^{lck}$ activity) is maintained below some critical level. One measure of this activity would be the accumulation of substrates bearing phosphotyrosine.

Increased Phosphotyrosine Content in pLGF Thymocytes. Immunoblotting studies performed using phosphotyrosine-specific antisera to examine pLGF thymocyte lysates revealed a very large number of substrates, all presumed targets for $p56^{lckF505}$-mediated phosphorylation (Fig. 8). The accumulation of phosphotyrosine-containing phosphoproteins in pLGF transgenic mice roughly paralleled transgene expression level (Table 1 and Fig. 2). Hence, there was a direct correlation between $p56^{lck}$ kinase activity, phosphotyrosine accumulation, and the extent of disruption observed in thymocyte maturation exhibited by *lck* transgenic mice.

Discussion

To investigate the functional importance of $p56^{lck}$, we have generated transgenic animals bearing both wild-type *lck*Y505 and mutant *lck*F505 expression constructs. This approach has several advantages over transfection of cloned T cell lines. First, expression was achieved in otherwise normal lymphocytes, obviating concerns about aberrant signaling pathways that might emerge in cells adapted to in vitro growth conditions. Second, expression of *lck* transgenes in the thymus permitted an analysis of the effects of augmented $p56^{lck}$ activity on thymocyte development, a process that is not yet amenable to in vitro dissection. Indeed, thymocyte maturation proved exquisitely sensitive to $p56^{lck}$ overexpression. Analysis of the

phenotypes of the pLGF and pLGY animals permits some inferences regarding the normal function of the *lck* gene and its product during thymopoiesis.

Dominant Control of lck Transgene Expression. By generating large numbers of founder animals, we observed that steady-state accumulation of pLGF (and pLGY) transgene mRNA is determined by the number of copies of the transgene integrated into host DNA. This phenomenon is unusual (44) but has been previously observed in at least two other gene systems: the β-globin genes (45) and, in a less thoroughly analyzed case, the CD2 gene (46). Importantly, globin constructs containing the dominant control region are expressed at levels comparable with the endogenous gene: a single transgene integrant, irrespective of the site of integration, yields steady-state transcript levels approximately equivalent to those derived from a single endogenous element.

The pLGF transgene behaves similarly in that transcript accumulation is copy number dependent. Hence, by this criterion, the pLGF transgene contains a dominant control region. However, it is difficult to compare *lck* transgene expression levels with those of the endogenous *lck* alleles, since both the proximal and distal *lck* gene promoters are simultaneously active in thymocytes (31, 42). Although extrapolations from our data suggest that 10 copies of the pLGF transgene are required to match transcriptions of the endogenous gene, the real value must be considerably lower. It is also possible that the pLGF transgene lacks additional positive regulatory elements found in the endogenous gene. In any case, identification of the *lck* dominant control region will clearly be of considerable interest, both in terms of elucidating the transcriptional regulation of the *lck* gene, and as a means of obtaining more efficient expression constructs for thymocytes and other lymphoid cells.

Augmented lck Expression Delays Thymocyte Maturation. The primary disturbance in pLGF mice is the loss of mature functional $CD3^+$ cells. When high levels of $p56^{lck}$ activity are present, the predominant cell phenotype in the thymus is $Thy\text{-}1^+$ and $CD3^-CD8^{lo}$ or $CD3^-CD8^-$. Decreased expression of the pLGF transgene permits appearance of more normal numbers of $CD3^+$ cells. Two lines of evidence support the contention that these disturbances are not the result of an artifactual property of activated $p56^{lckF505}$. First, in separate experiments, we have observed that overexpression of another *src* family kinase, $p59^{fyn}$, in transgenic animals under the control of the *lck* proximal promoter element yields thymocytes that exhibit a normal maturational sequence despite disruption of TCR-mediated signaling (49, 50). In addition, animals bearing transgenes encoding an activated form of an unrelated tyrosine kinase, *hck*, also exhibit no thymic development defects (data not shown). Second, and more persuasively, pLGY transgenic thymocytes, when compared with pLGF, show similar though less exaggerated abnormalities. In fact, assuming that $p56^{lckY505}$ and $p56^{lckF505}$ are equally stable, these proteins yield identical in vivo phenotypes at a ratio of $\sim$7:1 (Table 1). These results strongly support the view that $p56^{lckF505}$ acts qualitatively in the same fashion as its wild-type counterpart, but yields quantitatively superior effects.

Numerous possible explanations exist for the disrupted maturation of thymocytes in pLGF and pLGY mice. It is apparent, however, that $p56^{lck}$ activity crucially regulates thymocyte development. In fact, a simple doubling of the steady-state level of $p56^{lck}$ mRNA in the pLGY transgenics impairs the development of thymocytes with mature phenotypes (Table 1). It is attractive to view these results in the context of the known association of $p56^{lck}$ with the CD4 and CD8 coreceptor molecules. pLGF animals exhibit a transgene dose-dependent reduction in CD4 expression and simultaneously accumulate $CD8^{lo}$ cells in both the thymus and the periphery (Fig. 4). This observation suggests that thymopoiesis in pLGF animals is disrupted at the point where immature $CD3^-$ cells begin to acquire cell surface CD8 and CD4 (51). Consistent with this view, the thymocytes of pLGF animals contain few full-length TCR β chain transcripts, implying that maturation is disrupted at or near the time when V_β gene rearrangements occur (Fig. 6). Excessive $p56^{lck}$ activity may therefore provide a toxic or suppressive signal when coupled to appropriate cell surface receptors. In this context, it is important to note that CD8 α chains interact considerably less well with $p56^{lck}$ than do CD4 polypeptides, and that the CD8 β chain fails to associate with $p56^{lck}$ entirely (23). Hence, if $p56^{lck}$ delivers a suppressive or inhibitory signal during development in response to stimulation of CD4 and CD8 coreceptors, CD8 expression would be expected to be better tolerated in pLGF thymocytes. Regardless of the precise mechanism involved, it is clear that the level of activity of the *lck*-encoded protein tyrosine kinase must be strictly controlled in order for thymocyte development to proceed. Since even modest increases in $p56^{lck}$ abundance result in significant developmental disturbances, it is possible that alternations in *lck* expression or in $p56^{lck}$ structure may underlie some human immunodeficiency diseases.

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