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Development of T-leukaemias in CD45 tyrosine phosphatase-deficient mutant *lck* mice

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The CD45 tyrosine phosphatase lowers T-cell antigen receptor signalling thresholds by its positive actions on p56^{lck} tyrosine kinase function. We now show that mice expressing active *lck*^{F505} at non-oncogenic levels develop aggressive thymic lymphomas on a CD45$^{-/-}$ background. CD45 suppresses the tumorigenic potential of the kinase by dephosphorylation of the Tyr394 autophosphorylation site. In CD45$^{-/-}$ thymocytes the kinase is switched to a hyperactive oncogenic state, resulting in increased resistance to apoptosis. Transformation occurs in early CD4$^-$CD8$^-$ thymocytes during the process of TCR-β chain rearrangement by a recombinase-independent mechanism. Our findings represent the first example in which a tyrosine phosphatase *in situ* prevents the oncogenic actions of a *Src* family tyrosine kinase.

Keywords: apoptosis/CD45/lck/thymic lymphoma/tyrosine phosphatase

Introduction

The CD45 tyrosine phosphatase is expressed abundantly on all nucleated hematopoietic cells (Trowbridge and Thomas, 1994; Alexander, 1997). Earlier studies using CD45-deficient cell lines (Pingel and Thomas, 1989; Koretzky *et al*., 1990; Shiroo *et al*., 1992) and more recent results based on CD45$^{-/-}$ mice (Kishihara *et al*., 1993; Byth *et al*., 1996) have established a positive role for CD45 in controlling the signalling threshold of the T-cell antigen receptor (TCR), thereby regulating T-cell development (Alexander, 2000). Lymphoid precursors develop into mature TCR-αβ$^+$ T cells through a series of discrete maturation stages characterized by expression of CD4 and CD8 co-receptors (Rodewald and Fehling, 1998). Successful TCR-β rearrangements lead to expression of the pre-TCR on CD4$^-$CD8$^-$ (double-negative, DN) thymocytes, and the subsequent mediation of survival and proliferative signals in a process known as β-selection (Von Boehmer and Fehling, 1997). In CD45$^{-/-}$ mice there are two distinct defects in thymic development: a 2-fold reduction in the transition from DN to CD4$^+$CD8$^+$ (double-positive, DP) cells, and a 5-fold reduction in the further maturation of DP to single-positive CD4$^+$ and CD8$^+$ thymocytes (Kishihara *et al*., 1993; Byth *et al*., 1996). The CD45 thymocytes display increased basal apoptosis (Byth *et al*., 1996) and multiple defects in signals mediated by TCR-αβ (Stone *et al*., 1997) and by the pre-TCR (Pingel *et al*., 1999). The marked increase in the TCR signalling threshold in CD45$^{-/-}$ DP thymocytes perturbs both positive and negative selection (Mee *et al*., 1999).

Mutant CD45-deficient cell lines have been used to demonstrate that CD45 dephosphorylates the inhibitory C-terminal Tyr residues of both the p56^{lck} and p59^{fyn} tyrosine kinases (Ostergaard *et al*., 1989; Hurley *et al*., 1993; McFarland *et al*., 1993), consistent with their hyperphosphorylation in primary CD45$^{-/-}$ thymocytes (Stone *et al*., 1997). The CD4/CD8-associated p56^{lck} kinase in particular is critical for the phosphorylation of invariant Tyr-based motifs located within the cytoplasmic tails of CD3 and TCR-ζ polypeptides, thereby initiating TCR-mediated signalling cascades (Van Oers *et al*., 1996). In both lck$^{-/-}$ (Van Oers *et al*., 1996) and CD45$^{-/-}$ thymocytes (Stone *et al*., 1997) the tyrosine phosphorylation of TCR-ζ and CD3-ε is severely reduced. A model has therefore been proposed in which CD45-activated p56^{lck} acts to positively regulate TCR signal transduction by controlling the phosphorylation level of TCR polypeptides (Frearson and Alexander, 1997). However, this model has been questioned by the finding that in some CD45-deficient transformed T cell lines (Burns *et al*., 1994), as well as CD45$^{-/-}$ thymocytes (Doro and Ashwell, 1999), the average cellular p56^{lck} kinase activity is increased rather than decreased.

Formal demonstration that the C-terminal Tyr505 of p56^{lck} is a major physiologically relevant CD45 substrate in the T-lineage requires the reversal of the developmental defects noted in the CD45$^{-/-}$ mice by crossing with mice expressing an active form of p56^{lck}, in which this Tyr is mutated to Phe (Lck^{F505} mice). We (Pingel *et al*., 1999) and others (Seavitt *et al*., 1999) have recently carried out this experiment using mice expressing the mutant *lck* transgene under the regulation of the *lck* proximal promoter (Abraham *et al*., 1991a). Whereas high expression of the active *lck*^{F505} transgene alone causes thymic tumorigenesis (Abraham *et al*., 1991b), the low *lck* copy number mice used for back-crossing to CD45$^{-/-}$ mice do not develop tumours. However, they do display a transgene-dependent dose-related decrease in the production of TCR$^+$ thymocytes (Abraham *et al*., 1991a) due to the actions of mutant p56^{lck} in disrupting fruitful TCR-β chain rearrangements (Anderson *et al*., 1992; Lin and Abraham, 1997). As predicted, in CD45$^{-/-}$×Lck^{F505} mice there is a substantial restoration to near normal levels of

the transition from DN to DP thymocytes (Pingel *et al*., 1999). Furthermore, on a transgenic TCR background, CD4$^+$ mature T cells accumulate in the periphery and respond to antigen (Seavitt *et al*., 1999). Therefore, thymic development *in vivo* requires the dephosphorylation by CD45 of Tyr505 in the p56^{lck} C-terminus.

In the present work we describe the unexpected finding that all CD45$^{-/-}$×Lck^{F505} mice develop aggressive thymic tumours. Transformation is initiated at the stage of DN thymic development at which TCR-β chain rearrangement occurs, although tumours still develop on a *Rag-1*$^{-/-}$ background. Pre-tumorigenic CD45$^{-/-}$×Lck^{F505} thymocytes display increased resistance to apoptosis. Our results show that CD45 has a powerful suppressive effect on p56$^{lck–F505}$ activity in DN thymocytes by dephosphorylating its autophosphorylation site. In the absence of CD45, mutant p56$^{lck–F505}$ is converted to a hyperactive oncogenic form that promotes cell survival and bypasses the normal processes of β-selection. Our findings suggest that in wild-type mice CD45 exerts both positive and negative regulatory effects on p56^{lck} in the context of T cell development and function.

Results

CD45$^{-/-}$×Lck^{F505} mice develop thymic lymphomas by a recombinase-independent process

CD45 exon-9 targeted mice, which express no detectable CD45 (Byth *et al*., 1996), were crossed with two different mouse lines, pLGF-A (line 2964) and pLGF-C (line 3073), expressing 10.0 and 6.0 copies, respectively, of the active *lck^{F505}* transgene (Abraham *et al*., 1991a,b). Consistent with previous findings (Abraham *et al*., 1991a), we observed no thymic tumours in the pLGF-A or pLGF-C lines, nor were tumours detected in CD45$^{-/-}$ mice. In the CD45$^{-/-}$×pLGF-A and CD45$^{-/-}$×pLGF-C progeny, however, aggressive thymic tumours were observed in 100% of mice. Thymi bearing tumours were typically increased in cell number by 5-fold. All mice became ill and were sacrificed and necropsied. As Figure 1 illustrates, the earliest age of morbidity (5 weeks) and median survival time of 9.5 weeks were less for the higher *lck^{F505}* transgene-expressing CD45$^{-/-}$×pLGF-A progeny than for the CD45$^{-/-}$×pLGF-C progeny (cf. earliest morbidity of 10 weeks and median survival time of 12 weeks). These results are consistent with a dose-response relationship between *lck^{F505}* expression and tumorigenesis on the CD45 nullizygous background. Since tumour phenotypes were indistinguishable between CD45$^{-/-}$×pLGF-A and CD45$^{-/-}$×pLGF-C progeny, we chose to focus our investigation on the CD45$^{-/-}$×pLGF-A progeny, designated below as CD45$^{-/-}$Lck^{F505} mice.

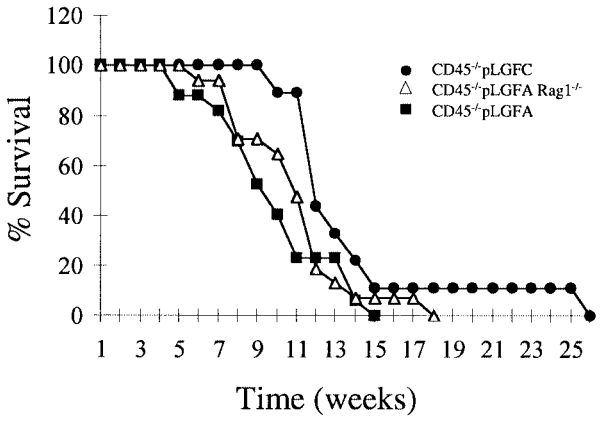


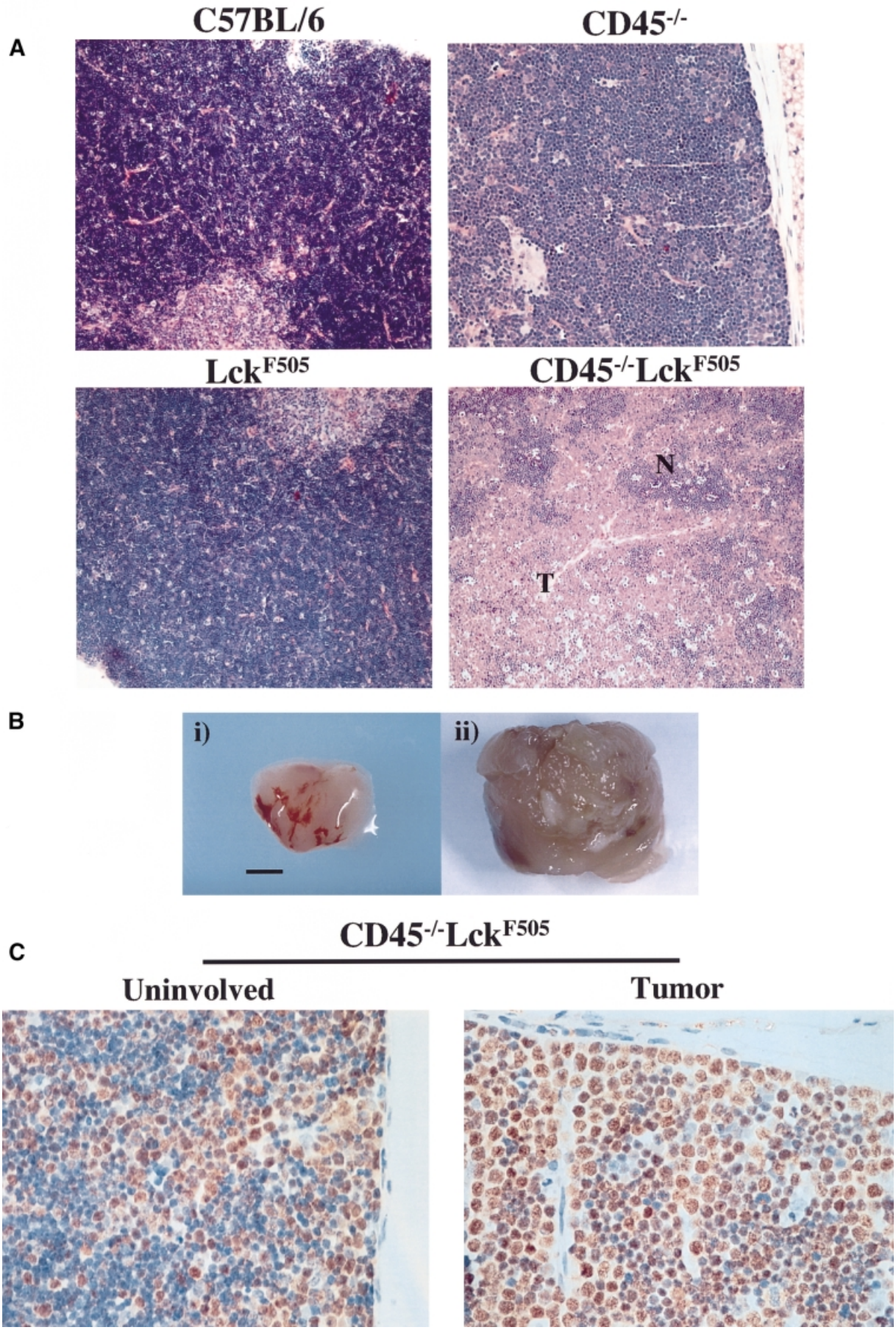
Fig. 1. Survival curves of CD45$^{-/-}$ mice expressing different copy numbers of the active *lck^{F505}* transgene showing Rag-1 independence of tumour development.

It has been suggested that aberrant V(D)J recombination and/or transpositional events may contribute to oncogenesis and some murine lymphomas are recombinase dependent (Hiom *et al*., 1998; Vanasse *et al*., 1999). We therefore crossed CD45$^{-/-}$Lck^{F505} mice with Rag-1$^{-/-}$ mice and investigated tumour development. The initiation of tumour development and median survival time of 10 weeks for CD45$^{-/-}$Lck^{F505}Rag-1$^{-/-}$ mice was indistinguishable from CD45$^{-/-}$Lck^{F505} mice (Figure 1). We therefore conclude that oncogenesis in CD45$^{-/-}$Lck^{F505} mice is recombinase independent.

As Figure 2A illustrates, histological analysis of thymic lymphomas from CD45$^{-/-}$Lck^{F505} mice (Figure 2B) revealed the 'starry sky' pattern characteristic of human high grade lymphomas (including lymphoblastic and Burkitt's types). This pattern is caused by numerous mitotic figures and tingible body macrophages, ingesting cellular debris, interspersed within a monotonous population of lymphoblasts. Extensive metastases were observed involving all other lymphoid and non-lymphoid organs investigated (spleen, liver, lungs and kidney; data not shown). Figure 2C shows a thymus from a 7-week-old mouse displaying a single lobe with a lymphoma apparently at an early stage of development. The lymphoma shows a marked increase in cells in cycle as revealed by the increase in the number of nuclei staining for the cell cycle-dependent minichromosome maintenance protein-2 (brown positive staining) (Freeman *et al*., 1999). In contrast, the uninvolved thymic lobe from the same mouse displays a proportion of cells in cycle comparable to wild type. These results show that lymphomas are initiated in discrete regions of a single thymic lobe and that mature tumours are characterized by increased apoptosis and extensive secondary metastases.

Thymic lymphomas develop from DN thymocytes undergoing TCR-β rearrangements

The DN thymic population, which comprises only 1–3% of the adult thymus, can be sub-divided into four discrete developmental stages according to the differential expression of CD44 (Pgp-1) and CD25 (IL-2 receptor α-chain). The order of development is: CD25$^-$CD44$^+$ (DN1), CD25$^+$CD44$^+$ (DN2), CD25$^+$CD44$^-$ (DN3) and CD25$^-$CD44$^-$ (DN4) (Rodewald and Fehling, 1998). Rearrangement of the TCR-β genes begins as cells exit from the DN2 stage and is completed at the DN3 stage. Successful β-selection requires p56^{lck}-dependent signals mediated by the pre-TCR (Von Boehmer and Fehling, 1997). Analysis of thymic development in CD45$^{-/-}$Lck^{F505} mice of different ages revealed profound abnormalities well before the appearance of overt lymphomas (Figure 3A). Increased side-scatter in FACS analyses showed that lymphoma cells were morphologically distinct in comparison with wild-type or non tumour-bearing


A
C57BL/6
CD45-/-
LckF505
CD45-/-LckF505
N
T
B
i)
ii)
C
CD45-/-LckF505
Uninvolved
Tumor

CD45$^{-/-}$Lck^{F505} thymocytes (Figure 3A, top panel). In contrast, analysis of two litter-mate CD45$^{-/-}$Lck^{F505} mice at 7 weeks revealed normal thymocyte numbers (Figure 3A, middle panel), a normal forward versus side-scatter profile (Figure 3A, top panel) and no tumour morphology. Nevertheless, thymocytes from one of the mice ('early') showed a striking increase in the DN population, whereas thymocytes from its sib ('none') appeared normal (Figure 3A, middle panel). The increase in DN cells was due to amplification of the DN3 sub-set (Figure 3A, bottom panel). In a fully developed tumour ('late'), 45% of the cells were in the DN compartment, the remaining cells displaying heterogeneous expression of CD4/CD8 (Figure 3A, middle and bottom panels). FACS analysis also revealed that the initiation of transformation in thymocytes from CD45$^{-/-}$Lck^{F505}Rag-1$^{-/-}$ mice appeared identical (data not shown). We therefore conclude that the recombinase-independent transforming event(s) that

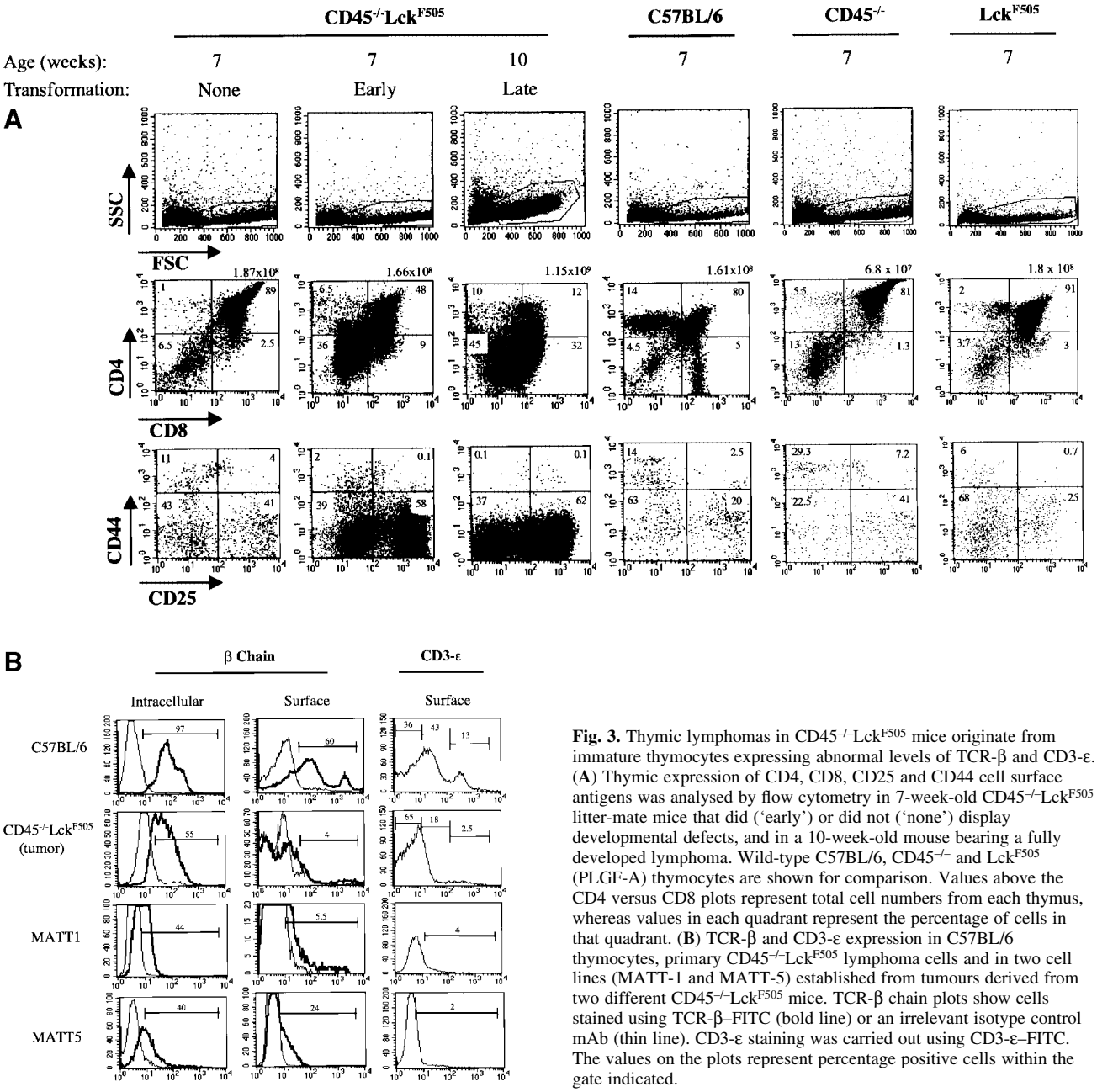


Fig. 3. Thymic lymphomas in CD45$^{-/-}$Lck^{F505} mice originate from immature thymocytes expressing abnormal levels of TCR-β and CD3-ε. (**A**) Thymic expression of CD4, CD8, CD25 and CD44 cell surface antigens was analysed by flow cytometry in 7-week-old CD45$^{-/-}$Lck^{F505} litter-mate mice that did ('early') or did not ('none') display developmental defects, and in a 10-week-old mouse bearing a fully developed lymphoma. Wild-type C57BL/6, CD45$^{-/-}$ and Lck^{F505} (PLGF-A) thymocytes are shown for comparison. Values above the CD4 versus CD8 plots represent total cell numbers from each thymus, whereas values in each quadrant represent the percentage of cells in that quadrant. (**B**) TCR-β and CD3-ε expression in C57BL/6 thymocytes, primary CD45$^{-/-}$Lck^{F505} lymphoma cells and in two cell lines (MATT-1 and MATT-5) established from tumours derived from two different CD45$^{-/-}$Lck^{F505} mice. TCR-β chain plots show cells stained using TCR-β–FITC (bold line) or an irrelevant isotype control mAb (thin line). CD3-ε staining was carried out using CD3-ε–FITC. The values on the plots represent percentage positive cells within the gate indicated.

Fig. 2. CD45$^{-/-}$Lck^{F505} mice develop thymic lymphoblastic lymphomas. (**A**) Hematoxylin and eosin-stained thymic sections from a 7-week-old mouse displaying representative early tumour development are shown with CD45$^{-/-}$, Lck^{F505} and C57BL/6 thymus for comparison. Early transformation events characteristically show lymphoblastic cells developing in foci (T) surrounded by normal tissue (N) often localized to one lobe of the thymus. (**B**) Comparison of normal (i) C57BL/6 and transformed (ii) CD45$^{-/-}$Lck^{F505} thymuses. The scale bar represents 5 mm. (**C**) A thymic tumour from a 7-week-old mouse in which proliferating (brown stain) lymphoblastic cells are amplified in one lobe ('tumor'), the other lobe ('uninvolved') displaying a wild-type morphology.

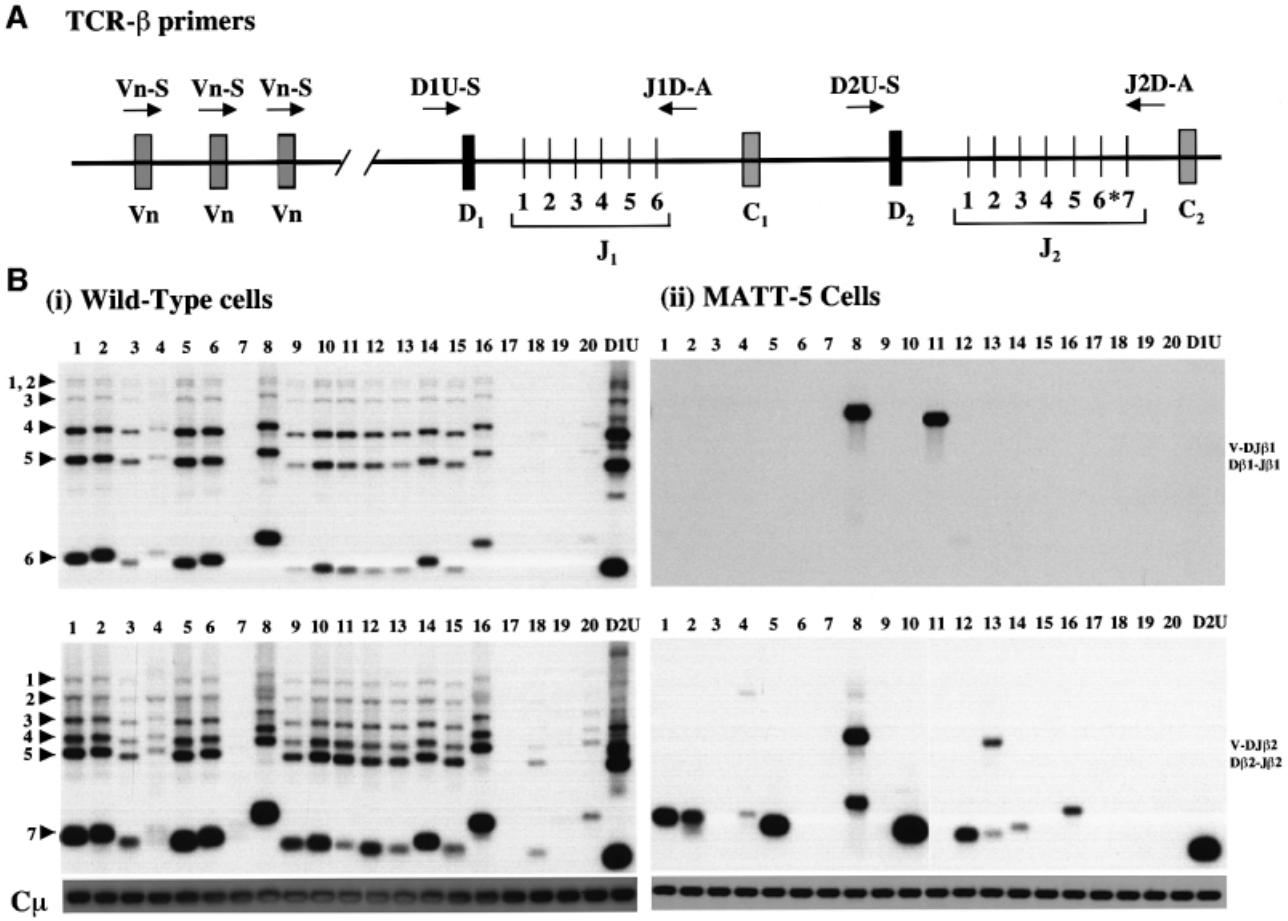


Fig. 4. Analysis of TCR-β chain rearrangements in CD45$^{-/-}$Lck^{F505} tumour cells. (**A**) Schematic representation of the murine TCR-β locus (based on Gärtner *et al*., 1999). Variable (V), diversity (D), joining (J) and constant region (C) segments are shown. Primers are indicated by arrows. The upstream primers are located either within (Vβ1–20) or immediately 5′ (D1U-S, D2U-S) of their target sequences. The downstream primers (J1D-A, J2D-A) are situated just 3′ of JB1.6 and JB2.7, respectively. The J-region Jβ2.6 marked with an asterisk is a pseudogene. (**B**) PCR analysis of D–J and V–DJ recombination in (i) wild-type C57BL/6 thymic DNA; (ii) MATT-5 tumour cell-line DNA. The rearrangement of Vβ and Dβ gene segments into the Jβ1 (upper panels) and Jβ2 (lower panels) regions was analysed using the PCR primers shown in (A). For each sample, the upstream primer used is shown at the top of the relevant track. For the downstream primers, J1D-A was used in both upper panels and J2D-A was used for both lower panels. Lanes D1U and D2U indicate Dβ1–Jβ1 and Dβ2–Jβ2 joining events, respectively. Lanes 1–20 indicate usage of Vβ1–20. Product specificity was confirmed by hybridization with oligonucleotide probes corresponding to Jβ1.6 and Jβ2.7 gene segments for the upper and lower panels, respectively (Gärtner *et al*., 1999). The bands marked by arrowheads represent rearrangements of Vβ genes to DJβ1 (upper panels) or DJβ2 (lower panels). Sequences from the Cμ gene were amplified as a loading control (lowest panels). Note that Vβ17 is a pseudogene in C57BL/6 mice. Prolonged exposure of gels revealed faint bands in the Vβ7 and Vβ19 lanes (lanes 7 and 19).

triggers abnormal cellular amplification and consequent tumour generation occurs during the DN3 stage of thymic development, the stage at which β-selection normally occurs.

The amplification of DN CD25$^{-/+}$CD44^{-} thymic lymphoma populations from 10-week-old CD45$^{-/-}$Lck^{F505} mice suggests that this 'pseudo-DN3' stage is attained by thoroughly subverting the normal process of β-selection. To facilitate further investigation of TCRαβ rearrangements, a series of MATT (murine active lck thymic tumour) cell lines was established from tumours found in different CD45$^{-/-}$Lck^{F505} mice. During early passages the cell lines were typically CD3^{lo}, CD4^{+}, CD8$^{+/-}$, Thy-1^{+}, CD25$^{+/-}$, CD44$^{+/-}$ and c-*kit*$^{-}$ (CD117). Interestingly, intracellular staining revealed that a significant proportion of lymphoma cells were TCR-β^{+}, and some TCR-β was also expressed at the cell surface (Figure 3B), indicating that some successful β-chain rearrangement had occurred, and excluding the possibility that cells were of NK cell origin. In contrast, intracellular staining with a panel of four different Vα-region mAbs showed that lymphoma cells were essentially negative for TCR-α, indicating that TCR-α rearrangements were blocked (data not shown). Lymphoma cells were also consistently negative for TCR-γδ, confirming an abortive commitment to the TCR-αβ lineage.

In normal thymic development, the assembly of TCR-β genes proceeds via a two-step process in which the initial joining of Dβ to Jβ segments on both chromosomes occurs first and is followed by Vβ to DJβ rearrangements (Malissen *et al*., 1992). To elucidate in more detail the extent of TCR-β gene rearrangements in lymphoma cells, we utilized a PCR approach previously used by others to demonstrate inhibition of TCR-β rearrangements in TCR-β or pLGF-A transgenic mice (Anderson *et al*., 1992; Ardouin *et al*., 1998; Gärtner *et al*., 1999). Figure 4 illustrates a representative result from a MATT tumour cell-line (Figure 4B, ii) showing a considerable reduction

in the extent of D–Jβ and V–DJβ recombination relative to normal cells (Figure 4B, i). Interestingly, no recombination event was noted in the Dβ1–Jβ1 region (Figure 4B, upper panel, lane D1U) despite clear evidence for recombination of Vβ8 and Vβ11 into this region (Figure 4B, ii, upper panel, lanes 8 and 11). This may represent a rare instance of direct Vβ to Jβ recombination. Comparable results were obtained from analysis of four primary tumours and two further MATT cell-line samples, which displayed unique or highly restricted DJ recombination events combined with a restricted Vβ repertoire (data not shown). Overall our results show that whereas tumour lineages are not clonal with respect to TCR-β rearrangement, they display restricted recombination events as compared with wild-type cells. In the case of MATT-5 tumour cells (Figure 4), the most economical interpretation is that transformation has occurred in a cell characterized by a single DJ joining event, but that subsequent V to DJ joining then continued in the daughter cells of this lineage utilizing a restricted range of Vβ genes.

Increased resistance to apoptosis in CD45$^{-/-}$Lck^{F505} thymocytes

We have shown previously that CD45$^{-/-}$ thymocytes display a marked increase in basal apoptosis (Byth *et al*., 1996; Conroy *et al*., 1997). Annexin V staining provides a convenient method for assessing the early stage of apoptosis at which an increased level of phosphatidylserine is expressed in the outer leaflet of the plasma membrane. Figure 5A shows that the percentage of annexin V^{hi} CD45$^{-/-}$ DP cells is increased ~4-fold, and the mean level of annexin V staining intensity ~7-fold, when compared with wild-type or pLGF-A DP thymocytes, consistent with our previous results. In contrast there was no obvious increase in annexin V staining in gated CD45$^{-/-}$ DN cells (Figure 5A), although we cannot exclude the possibility that increased basal apoptosis occurs in a specific DN1–DN4 subset too small to detect by this method. In pre-tumour CD45$^{-/-}$Lck^{F505} DP thymocytes there was a marked 3-fold reduction in annexin V intensity when compared with CD45$^{-/-}$ DP thymocytes, reflecting increased resistance to apoptosis in the presence of the active *lck^{F505}* transgene (Figure 5A). To confirm that the increased resistance to apoptosis was a direct consequence of the active *lck^{F505}* transgene and not caused indirectly by the initiating processes of transformation, we retrovirally transduced CD45$^{-/-}$ fetal thymus organ cultures (FTOC) with active *lck^{F505}* cDNA and determined the effect on thymic apoptosis after 7 days, a period too short for oncogenesis to occur. Figure 5B shows that p56$^{lck–F505}$ expression caused a marked reduction in basal apoptosis in CD45$^{-/-}$ thymocytes (from 50 to 15% annexin v^{hi} cells), in agreement with the results obtained using thymocytes prepared directly from CD45$^{-/-}$Lck^{F505} mice. FACS analysis revealed no gross changes in thymic subsets following retroviral transduction, which could explain these changes in annexin V staining (data not shown). However, p56$^{lck–F505}$ expression causes the accumulating DN3 cells in CD45$^{-/-}$ FTOC to transit through to the DN4 stage in agreement with our previous results using thymocytes from CD45$^{-/-}$Lck^{F505} mice (Pingel *et al*., 1999).

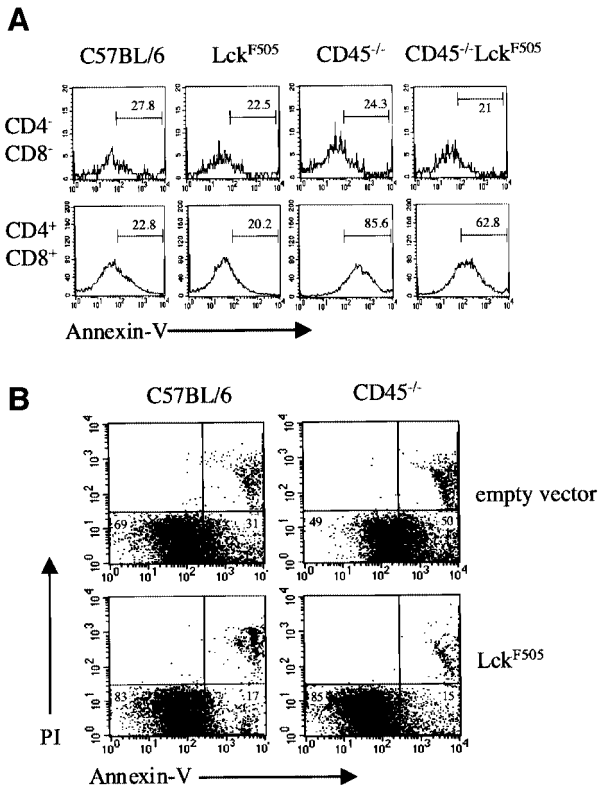


Fig. 5. p56$^{lck–F505}$ reduces the high level of basal apoptosis in CD45$^{-/-}$ thymocytes. (**A**) Electronically gated CD4-PE and CD8-biotin DN and DP thymocytes from C57BL/6, pLGFA, CD45$^{-/-}$ and pre-tumorigenic CD45$^{-/-}$Lck^{F505} mice were analysed for expression of annexin V–FITC by flow cytometry. The percentage of cells within each gate is indicated. (**B**) Retroviral transduction of active *lck^{F505}* cDNA or empty vector into FTOC derived from C57BL/6 or CD45$^{-/-}$ mice. Apoptosis was determined by staining with annexin V and PI; the values represent the portion of annexin V staining cells (excluding PI positive cells).

Hyperactive p56$^{lck–F505}$ kinase in CD45$^{-/-}$ thymocytes greatly increases protein tyrosine phosphorylation

The expression level of p56$^{lck–F505}$ in the pLGF-A mouse line is insufficient to cause thymic lymphomas. However, on a CD45$^{-/-}$ background dramatic changes occur that result in a marked increase in resistance to apoptosis (Figure 5) and developmental abnormalities (Figure 3) resulting in thymic lymphomas with ages of onset related to the dose of the active *lck^{F505}* transgene (Figure 1). In principle, CD45 nullizygosity could perturb a signalling pathway quite distinct from that mediated by p56$^{lck–F505}$, resulting in synergistic effects leading to transformation. Alternatively the actions of p56$^{lck–F505}$ might be directly amplified in the absence of CD45. To distinguish these possibilities biochemical studies were performed using pre-tumour CD45$^{-/-}$Lck^{F505} thymocytes. Figure 6A (upper panel) shows that p56^{lck} was hyperphosphorylated in these cells compared with the kinase immunoprecipitated from wild-type or pLGF-A thymocytes. The p56^{lck} kinase activity, as measured by autophosphorylation, was also 2-fold elevated in comparison with wild-type thymocytes (Figure 6B). In contrast, the activities of p56^{lck} isolated from pLGF-A or CD45$^{-/-}$ thymocytes revealed only modest increases. Only when the active *lck* transgene

was expressed on a CD45 nullizygous background was hyperactivity detected. The p56^{lck} hyperphosphorylation illustrated in Figure 6A could theoretically occur at either the Tyr394 autophosphorylation site and/or the Tyr505 inhibitory regulatory site in the endogenous p56^{lck} or at Tyr394 in the p56$^{lck–F505}$ kinase expressed from the mutant transgene. To distinguish these possibilities thymocyte lysates were immunoblotted with a phosphopeptide antibody specific for the autophosphorylation site of Src, previously shown to cross-react with the equivalent Tyr394 site in p56^{lck} (Hardwick and Sefton, 1997). Interestingly, Tyr394 phosphorylation in p56^{lck} from CD45$^{-/-}$Lck^{F505} thymocytes was increased by 52 and 64%, respectively, when compared with wild-type and pLGF-A thymocytes (Figure 6A, lower panel). A smaller (23%) increase in Tyr394 phosphorylation in p56^{lck} from CD45$^{-/-}$ thymocytes was also detected in comparison with wild type. Since there was no evidence for increased autophosphorylation in p56^{lck} from the CD45$^{+/+}$ pLGF-A thymocytes that have the same ratio of p56$^{lck–F505}$ to endogenous p56^{lck}, it is apparent that p56$^{lck–F505}$ Tyr394 from pLGF-A thymocytes is dephosphorylated by CD45, but in CD45$^{-/-}$ thymocytes this site becomes hyperphosphorylated (Figure 6A), so increasing the kinase activity (Figure 6B). In fact Figure 6C and D shows that in detergent-soluble and -insoluble fractions, respectively, from CD45$^{-/-}$Lck^{F505} thymic lysates, there is a striking increase in protein tyrosine phosphorylation compared with controls, consistent with hyperactivity of the p56$^{lck–F505}$ kinase. Whereas numerous proteins displayed basally elevated phosphotyrosine levels in the detergent-soluble fraction, in the insoluble fraction containing cytoskeletal proteins and cholesterol-enriched lipid rafts, two major hyperphosphorylated proteins were identified: a p56 protein identified as p56^{lck} (data not shown) and a p80–85 kDa protein that remains unidentified. In four separate experiments there were average 30- and 11-fold increases in p80–85 and p56^{lck} tyrosine phosphorylation, respectively, in the detergent-insoluble fraction, whereas p56^{lck} protein was increased 2.3-fold compared with wild type, so the p56^{lck} present in this fraction is also hyperphosphorylated. It has previously been reported that a portion of activated p56^{lck} associates with the cytoskeleton in T cells (Rozdzial *et al*., 1998) and it seems likely, therefore, that in CD45$^{-/-}$Lck^{F505} thymocytes a pool of active p56^{lck} is re-localized to the cytoskeleton under basal conditions. As Figure 6E illustrates, a high basal level of tyrosine phosphorylation was also observed in MATT cell lines in comparison with normal thymocytes. Interestingly, phosphorylation was further elevated upon ligation of the CD3 complex and/or pre-TCR with a CD3 mAb (Figure 6E, upper panel), and immunoblotting for p56^{lck} protein revealed an increase in the p59^{lck} characteristic of activated T cells (Figure 6E, lower panel), demonstrating that the low level of CD3/pre-TCR expressed on these cells (cf. Figure 3B) can be coupled to intracellular signalling pathways. We also compared the p56^{lck} kinase activities between MATT-6 cells and the 2047F cell line (Lin and Abraham, 1997) derived from a tumour from a CD45$^{+/+}$ mouse (no. 1127), expressing mutant lck^{F505} at higher copy number than the PLGF-A mice (Abraham *et al*., 1991b). As expected, 2047F cells displayed increased (48% higher) p56^{lck} expression compared with MATT-6 cells (Figure 6F). However, the p56^{lck} kinase activity, normalized for kinase protein, was 35% lower in 2047F compared with MATT-6 cells (mean of four separate assays; see Figure 6F for a representative result), consistent with the hypothesis that CD45 inhibits p56$^{lck–F505}$ kinase activity in 2047F cells.

We conclude that in CD45$^{+/+}$ thymocytes expressing p56$^{lck–F505}$, CD45 dephosphorylates the Tyr394 autophosphorylation site, thereby suppressing its activation. In the absence of CD45 the kinase becomes hyperactive, resulting in increased protein tyrosine phosphorylation in critical substrates mediating survival signals leading, eventually, to transformation.

Discussion

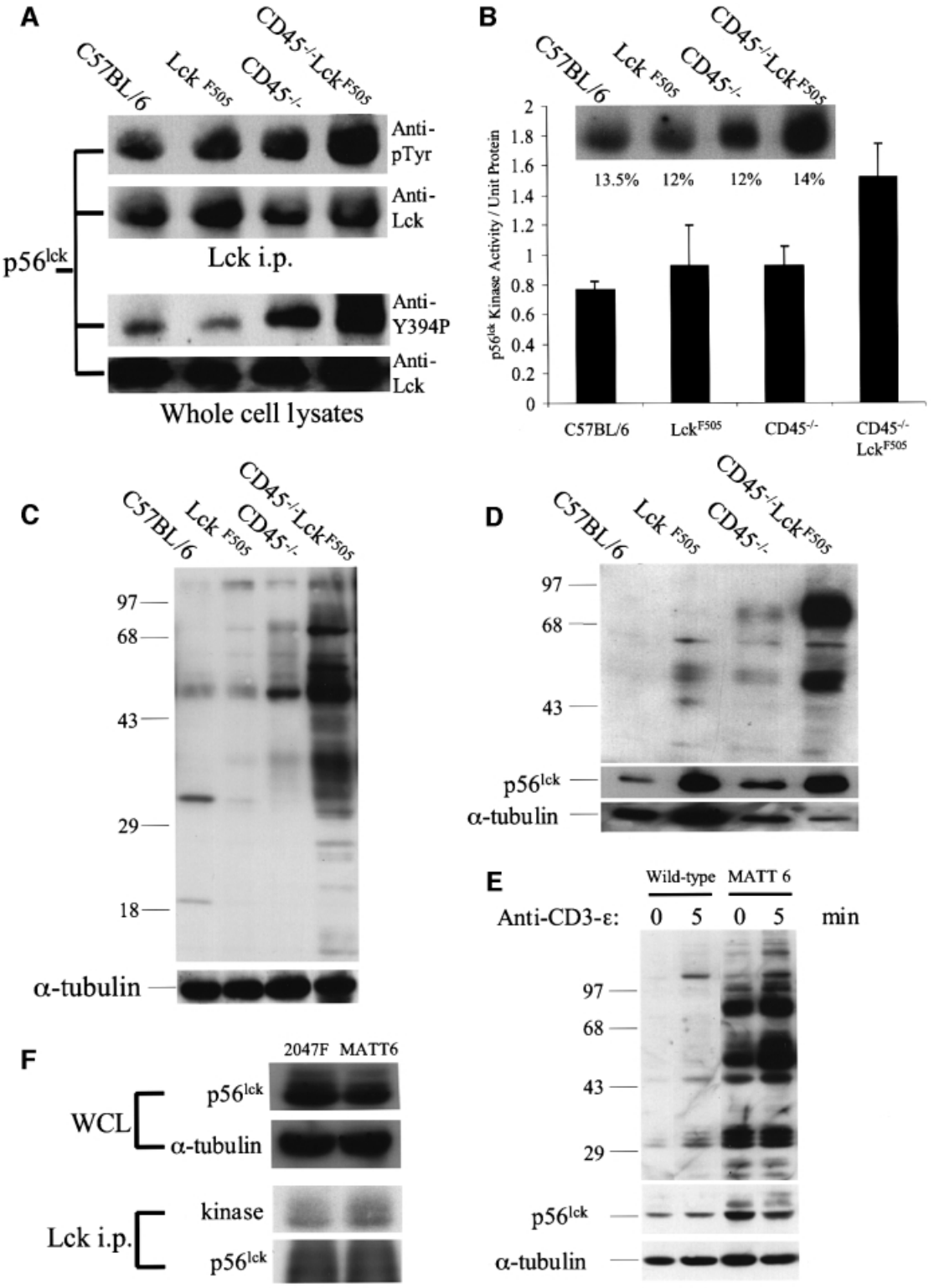
CD45 suppresses the tumorigenic potential of p56$^{lck–F505}$

Our findings reveal an unexpected role for the CD45 tyrosine phosphatase as a negative regulator of the transforming actions of p56$^{lck–F505}$. In the pLGF-A and pLGF-C mouse lines, which express the transgene at relatively low levels (copy number 6–10), p56$^{lck–F505}$ prevents CD3/TCR expression and triggers maturational arrest by inducing premature allelic exclusion (Abraham *et al*., 1991a; Anderson *et al*., 1993). Only when the active *lck^{F505}* transgene reaches a copy number >23, or wild-type p56^{lck} is expressed at very high levels (copy number of 70), do thymic lymphomas then develop at 8 weeks in addition to the early maturational dysfunction (Abraham *et al*., 1991a).

Our results show that non-tumorigenic p56$^{lck–F505}$ is converted into an oncogene by CD45 nullizygosity

Fig. 6. Hyperactive p56^{lck} kinase in CD45$^{-/-}$Lck^{F505} thymocytes induces increased protein tyrosine phosphorylation. All experiments were performed using pre-tumorigenic CD45$^{-/-}$Lck^{F505} thymocytes. (**A**) Immunoprecipitated p56^{lck} was immunoblotted for phosphotyrosine (upper panels) and whole cell lysates were probed with an antibody recognizing p56^{lck} pTyr394 (lower panels). Immunoblots were reprobed for p56^{lck} to show comparable protein loading. (**B**) p56^{lck} *in vitro* kinase activity is increased in CD45$^{-/-}$Lck^{F505} thymocytes. The insert illustrates phosphorylation levels in a representative experiment and the values shown are relative phosphorimager values obtained by reprobing for p56^{lck} protein. The bar chart represents mean data ± SD from three independent experiments. Kinase values have been normalized for protein loading. The CD45$^{-/-}$Lck^{F505} values are significantly different (P <0.05) (Student's t-test) from all other values shown. (**C**) Protein tyrosine phosphorylation is increased in CD45$^{-/-}$Lck^{F505} thymic lysates. The blot was reprobed for α-tubulin to show equal loading of protein. (**D**) The tyrosine phosphorylation of 56 and 80–85 kDa proteins in the detergent insoluble fraction of CD45$^{-/-}$Lck^{F505} thymocytes. The blot was reprobed for p56^{lck} and for α-tubulin to show protein loading. Note that slight under-loading in the CD45$^{-/-}$Lck^{F505} track compared with C57BL/6 implies that both protein hyperphosphorylation and the p56^{lck} protein level are greater than illustrated. See text for quantification of mean values. (**E**) The MATT-6 cell line established from a CD45$^{-/-}$Lck^{F505} tumour and wild-type thymocytes as control were stimulated with CD3-ε (2C11) mAb for the times indicated. The blot was reprobed for p56^{lck} (middle panel), and for α-tubulin (bottom panel) to show comparable loading of protein. (**F**) Whole-cell lysates from MATT-6 (3×10^7) and 2047F (2×10^7) cells were probed for p56^{lck} and α-tubulin (upper panels) and *in vitro* kinase assays were carried out in p56^{lck} immunoprecipitates, which were subsequently probed for p56^{lck} protein (lower panels). See text for quantification.

(Figure 1) and reveal a molecular mechanism for the initiation of transformation. PLGF-A thymocytes display few signs of p56$^{lck–F505}$ hyperactivity compared with wild type whereas on a CD45$^{-/-}$ background the same level of p56$^{lck–F505}$ shows hyperactivation (Figure 6). Our results strongly suggest that in pLGF-A mice CD45 suppresses activity of the p56$^{lck–F505}$ kinase by dephosphorylating the Tyr394 autophosphorylation site, a regulatory phosphotyrosine already known to play a dominant role in promoting the kinase activity of Src family kinases (Doro *et al*., 1996). CD45 could dephosphorylate other phosphorylated substrates downstream of p56^{lck}, which in turn contribute to oncogenesis by becoming hyperphosphorylated in CD45$^{-/-}$ thymocytes. However, considering that high p56$^{lck–F505}$ levels are intrinsically tumorigenic (Abraham *et al*., 1991a), the hyperactivation of low-level



$p56^{lck–F505}$ that occurs on a $CD45^{-/-}$ background appears both necessary and sufficient to explain oncogenesis. Presumably in the $CD45^{+/+}$ context, when the mutant or wild-type $p56^{lck}$ levels become very high, the actions of CD45 are insufficient to maintain all the kinase molecules dephosphorylated at Tyr394, resulting in increased activity and consequent transformation.

Our results are consistent with previous data using transformed T- and B-cell lines showing that CD45 can function to dephosphorylate the autophosphorylation sites of $p56^{lck}$ (Doro *et al*., 1996) and $p59^{lyn}$ (Katagiri *et al*., 1999), respectively, besides the C-terminus inhibitory phosphorylation sites of these kinases. Presumably other tyrosine phosphatases play comparable roles in dephosphorylating the autophosphorylation sites of other Src family members, such as v-Src, thereby suppressing the oncogenic potential of these kinases.

Apoptosis and the initiation of transformation

Transformation in $CD45^{-/-}Lck^{F505}$ mice appears to be initiated at the DN3/DN4 stage of thymic differentiation at which TCR-β chain rearrangements occur (Figure 3A). At this early stage of tumorigenesis, a large increase in the proportion of cycling cells was revealed histologically (Figure 2C), presumably representing the DN $CD44^{-}$ populations detected by flow-cytometric analysis (Figure 3A). In contrast to pLGFA mice (Anderson *et al*., 1992), successful β-chain rearrangements occurred in $CD45^{-/-}Lck^{F505}$ lymphoma cells (Figure 4) resulting in some expression of the TCR-β chain (Figure 3B). Extensive chromosomal damage involving trisomies and translocation of known oncogenes was also noted in MATT cell-lines (data not shown). Tumour initiation during V(D)J recombination might therefore support the hypothesis that recombinase-induced DNA damage, including the translocation and activation of proto-oncogenes, contributes to oncogenesis (Hiom *et al*., 1998; Vanasse *et al*., 1999). However, tumour development was independent of recombinase actions (Figure 1), excluding this supposition in the present instance. $CD45^{-/-}Lck^{F505}$ thymic lymphomas are therefore similar in this respect to $p53^{-/-}$ lymphomas, which also develop on a $Rag\text{-}1^{-/-}$ background (Liao *et al*., 1998; Nacht and Jacks, 1998).

Interestingly, non-tumorigenic levels of the active lck^{F505} transgene appear necessary and sufficient to initiate all of the critical events controlled by the pre-TCR at the DN/DP transition, including survival, onset of proliferation, acquisition of CD4/CD8 and inhibition of TCR-β rearrangements (Anderson *et al*., 1992; Mombaerts *et al*., 1994; Haks *et al*., 1999; Pingel *et al*., 1999). Therefore, hyperactive oncogenic $p56^{lck–F505}$ must mediate signals additional to those necessary for the DN/DP transition. It has been suggested that in wild-type thymocytes, V(D)J rearrangements induce p53 activation and cell cycle arrest, and that subsequent pre-TCR signalling then causes p53 inactivation, thereby mediating survival and release from cell cycle arrest (Jiang *et al*., 1996; Haks *et al*., 1999). One possibility is that hyperactive $p56^{lck–F505}$ subverts this normal sequence of events, promoting survival (Figure 5) and driving DN3/DN4 cells into cycle, as we in fact observe (Figures 2C and 3A), irrespective of whether or not successful TCR-β chain rearrangement has occurred. Presumably only at the DN/DP transition can hyperactive $p56^{lck–F505}$ disrupt the fine balance between apoptosis and cell cycle progression to trigger transformation.

Tumour development in $CD45^{-/-}Lck^{F505}$ mice raises the question of the possible relevance of this mouse model to an understanding of human oncology. Despite earlier interest, with rare exceptions (Wright *et al*., 1994), mutations of the *lck* gene have not been described in human cancers. In contrast we have previously described a patient with T-acute lymphoblastic leukaemia whose T-lineage cells were substantially CD45-negative (Biffen *et al*., 1994), and others have described the more common finding that CD45 expression on B-lineage lymphoblastic leukaemia cells may be absent or much reduced (Behm *et al*., 1992; Borowitz *et al*., 1997; Vormoor *et al*., 1998). The possibility that CD45-loss plays a role in the transformation of human lymphocytes deserves further investigation.

CD45 as a negative regulator of $p56^{lck}$

The contrasting positive and negative roles of CD45 in the regulation of T-cell signalling and development has attracted considerable attention (Ashwell and Doro, 1999; Thomas, 1999; Thomas and Brown, 1999; Alexander, 2000). The present work establishes that CD45 can act as a negative regulator of $p56^{lck–F505}$ *in situ*. This provokes the question as to whether CD45 exerts a similar action on the wild-type $p56^{lck}$ molecule. That this is the case is suggested by the increased $p56^{lck}$ kinase activity in $CD45^{-/-}$ thymocytes that we (Stone, 1996) and others (Doro and Ashwell, 1999) have reported previously, and by our present observation that $p56^{lck}$ autophosphorylation is increased in $CD45^{-/-}Lck^{F505}$ compared with wild-type thymocytes (Figure 6A). We have also previously reported that in $CD45^{-/-}$ thymocytes the proportion of hyperphosphorylated $p56^{lck}$ in its 'closed' conformation is increased, indicating that much of the phosphate is at the inhibitory C-terminus Tyr505 (Stone *et al*., 1997). These disparate findings are readily reconciled by a model in which CD45 can dephosphorylate both the Tyr394 and Tyr505 $p56^{lck}$ phosphorylation sites *in situ* (Alexander, 2000). The average $p56^{lck}$ activity in $CD45^{-/-}$ cell lysates is expected to be higher due to the dominant effect of $p56^{lck}$ molecules phosphorylated at Tyr394 (Doro *et al*., 1996), even though the majority of $p56^{lck}$ molecules are thought to be phosphorylated at Tyr505, thereby inhibiting engagement of their SH2 domains with exogenous proteins (Stone *et al*., 1997; Alexander, 2000). Which type of phosphorylated molecule predominates in the CD4/CD8-associated pool of $p56^{lck}$, which is critical for the initiation of normal TCR signalling, remains to be resolved, although our previous findings suggest that this is a low-activity pool (Biffen *et al*., 1994). A dominantly positive role for CD45 in the T-lineage is indicated by the high threshold for pre-TCR and TCR signalling apparent in $CD45^{-/-}$ T cells (Kishihara *et al*., 1993; Byth *et al*., 1996; Stone *et al*., 1997; Mee *et al*., 1999; Pingel *et al*., 1999) and by the ability of $p56^{lck–F505}$ to rescue the $CD45^{-/-}$ phenotype (Pingel *et al*., 1999; Seavitt *et al*., 1999).

In summary, the active *lck* transgene expressed at low levels is non-oncogenic due to the suppressing actions of CD45. The CD45 nullizygous phenotype is likewise non-

oncogenic, presumably due to the hyperphosphorylation of Tyr505, which maintains most of the p56^{lck} in a 'closed conformation' signalling incompetent state. In contrast the active *lck* transgene combined with CD45 nullizygosity is aggressively oncogenic. Our findings provide the first example in which an identified tyrosine phosphatase *in situ* has been shown to suppress the oncogenic actions of a Src family kinase.

Materials and methods

Mice

Mice were maintained under specific pathogen free conditions in the animal facility at The Babraham Institute, Cambridge, UK. Mice deficient for Rag-1 and CD45 have been described in detail elsewhere (Spanopoulou *et al*., 1994; Byth *et al*., 1996; Pingel *et al*., 1999). The pLGF-A (line 2964) and pLGF-C (line 3073) transgenic mice expressing constitutively active p56$^{lck–F505}$ under the control of the lck proximal promoter have been previously described (Abraham *et al*., 1991a,b). Transgene positive mice were determined by the presence of a 150 bp PCR fragment unique to the mutant transgene using primers: pLGFA fwd ATGACTTCTTCACAGCCACAGAGGG and pLGFA rvs TTTTAT-TAGGACAAGGCTGGTGGGC.

Flow cytometry

Preparation of samples for flow cytometry was performed as described (Pingel *et al*., 1999). For intracellular staining, cells were washed twice in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min at 4°C. Cells were washed once in 200 µl of PBA [1× PBS, 1% bovine serum albumin (BSA) and 0.02% NaN_3] and treated with 100 µl of 0.5% Saponin for 15 min at 4°C. Antibodies diluted in 0.5% Saponin were used to stain cells for 30 min at 4°C followed by washing in 200 µl of PBA. Finally, cells were analysed on a Becton-Dickinson FACScalibur. Biotin- or FITC-conjugated antibodies specific for CD3-ε (clone 145-2C11), TCR-β (clone H57-597), Vα8 (clone B21.14), Vα11.1 + 11.2^{b,d} (clone RR8-1), Vα3.2^{b,c} (clone RR3-16) and Vα2 (clone B20.1) were obtained from PharMingen. Biotin- or PE-conjugated anti-mouse CD4, CD8, CD25 and CD44 were purchased from Caltag. FITC-isotype control antibodies: hamster IgG group 2λ, rat IgG_1κ, rat IgG_{2b}κ and rat IgG_{2a}κ were purchased from PharMingen. Where appropriate, streptavidin-Tricolor (Caltag) was used as a second-step reagent.

Staining of paraffin-embedded tissues

Immunochemistry was performed as previously described (Freeman *et al*., 1999). Mouse mAb against Mcm2 (BM28) was purchased from Transduction Laboratories (Lexington, KY).

Cell lines

The 2047F cell line (Lin and Abraham, 1997) derived from a tumour from a CD45$^{+/+}$ mouse expressing the *lck*^{F505} transgene (no. 1127) (Abraham *et al*., 1991b) was kindly provided by Dr K.Abraham (University of Maryland, MD). MATT cell lines were generated by seeding disaggregated thymic lymphoma cells on to γ-irradiated MRC-5 cells and culturing in RPMI/20% FCS with 50 µM mercaptoethanol and supplementary MEM non-essential amino acid solution (Sigma). Once cells were established in culture they were removed from MRC-5 cells and the FCS was reduced to 10%. No cytokine addition was necessary for optimal growth.

Analysis of TCR-β gene rearrangements

Analysis of TCR-β chain gene rearrangement in genomic DNA using a PCR assay was performed essentially as described previously (Gärtner *et al*., 1999). The PCR primers and probes are described in the legend to Figure 4.

Apoptosis

Flow cytometric analysis of apoptotic cells was performed using annexin V and propidium iodide (PI). Cells were stained for surface markers and washed once in PBS. The equivalent of 1×10^6 cells were resuspended in 100 µl of annexin V binding buffer (2.5 mM $CaCl_2$, 10 mM HEPES pH 7.4 and 140 mM NaCl) and cells were incubated in the dark with 2.5 µg/ml PI and 3 µl of annexin V–FITC for 15 min at room temperature.

Retroviral transduction into fetal thymus organ culture

Retroviral transduction was carried out as described previously (Crompton *et al*., 1996). Foetal thymi were dissected on day E14 and incubated for 72 h in Terasaki wells containing stably transfected adherent retroviral packaging cells at 1×10^5 cells/ml. Lobes were transferred to Millipore filters and incubated for a further 6 days prior to analysis.

Immunoblotting and immunoprecipitation

Cells were either prepared in 1% NP-40 lysis buffer with 1 mM Na_3VO_4 as described previously (Pingel *et al*., 1999) or whole-cell extracts were generated by adding SDS–PAGE sample buffer (2% SDS, 10% glycerol, 80 mM Tris–HCl pH 6.8). Proteins were separated by 7–15 or 4–20% SDS–PAGE and transferred to Immobilon-P. Immunoblots were probed with antibodies to phosphotyrosine (4G10, Upstate Biotechnology Inc.), p53 (CM5, Novocastra), α-tubulin (DM 1A, Sigma), Src-pY^{418} (Biosource International) (Osusky *et al*., 1995) and p56^{lck} (688, kind gift of L.Samelson). For immunoprecipitation, protein G–Sepharose beads were coated with specific antibodies and incubated with pre-cleared cell lysates (generated in 1% NP-40 lysis buffer from 6×10^7 cells) for 2 h at 4°C before washing three times in 1% NP-40 lysis buffer. For *in vitro* kinase assays p56^{lck} was immunoprecipitated and washed three times in 1% NP-40 lysis buffer followed by washing once in kinase buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$). The p56^{lck} precipitate was resuspended in 30 µl of kinase buffer with 5 µM ATP and 2–4 µCi [γ-^{32}P]ATP and incubated at room temperature on a shaker for 20 min. The reaction was stopped by adding 5 µl of 6× sample buffer and boiling for 5 min. Proteins were separated by 7–15% SDS–PAGE and immunoblotted onto Immobilon-P. Bands were visualized by autoradiography.

Quantitation of chemiluminescent and radioactive samples was performed using a Bio-Rad phosphorimager (Hemel Hempstead, UK).

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