of ectopic activity in these neurons and/or other contributory mechanisms.

The nerve damage that precipitates neuropathic pain leads to many changes in sensory neurons, including alterations in putative neurotransmitters/modulators, receptors, ion channels, structural proteins, and anatomic terminations (*21*). The relative contributions of these reactive changes are currently unknown, especially the role of small-caliber nociceptive neurons or large-caliber mechanosensitive afferents (*22*). There is conflicting evidence regarding the role of nociceptors. Antisense treatment against SNS (normally expressed in nociceptors) reduces neuropathic pain behavior (*23*). However, few unmyelinated afferents discharge ectopically in neuropathic models (*24*), and mechanical hyperalgesia is unaffected by the C fiber toxin RTX (*25*). We have also found that the development of neuropathic pain behavior is unaffected in null mutant mice lacking the SNS channel (*26*). In contrast, the data here and in the literature support a pivotal role for myelinated afferents in the generation of neuropathic pain: animal models show that an essential drive for abnormal pain sensitivity is the generation of ectopic activity in damaged sensory neurons (*12*, *27*), arising almost exclusively in myelinated neurons; in human neuropathic pain states, activation of large Aβ afferents is capable of inducing pain (*28*); selective lesions of large myelinated afferents reduce neuropathic pain behavior in animals (*29*).

The ectopic activity that arises in neuropathic conditions is TTX-sensitive. Only the expression of the TTX-sensitive type III α subunit is known to increase following nerve injury (*20*), and we show here that GDNF prevents this. It is unclear whether GDNF acts tonically to repress type III expression under normal circumstances. As GDNF treatment does not affect pain-related behavior in normal animals, the analgesic actions reported here are unlikely to represent general effects on pain signaling systems. However, as GDNF regulates the expression of a variety of genes in both large- and small-caliber sensory neurons, including some functionally relevant for nociceptive behavior [P2X3 and VR1 (*6*, *7*)], effects other than those on the type III sodium channel may contribute to its analgesic actions. This question can only be definitively addressed with specific, type III channel blockers, which have yet to be developed. However, the data presented here provide a rational basis for, and demonstrate the efficacy of, GDNF in the treatment of neuropathic pain.

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Long-Term Survival But Impaired Homeostatic Proliferation of Naïve T Cells in the Absence of p56^{lck}

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Interactions between the T cell receptor (TCR) and major histocompatibility complex antigens are essential for the survival and homeostasis of peripheral T lymphocytes. However, little is known about the TCR signaling events that result from these interactions. The peripheral T cell pool of p56^{lck} (lck)–deficient mice was reconstituted by the expression of an inducible lck transgene. Continued survival of peripheral naïve T cells was observed for long periods after switching off the transgene. Adoptive transfer of T cells from these mice into T lymphopoienic hosts confirmed that T cell survival was independent of lck but revealed its essential role in TCR-driven homeostatic proliferation of naïve T cells in response to the T cell–deficient host environment. These data suggest that survival and homeostatic expansion depend on different signals.

Despite environmental antigenic stimulation and thymic production, the size of the peripheral T cell pool is maintained at a remarkably constant level (*1*). In common with cells of other tissues, T cells require specific signals in order to survive. In contrast to memory T cells (*2–4*), naïve T cells require interactions of the TCR with self major histocompatibility complex (MHC) antigens for their prolonged survival (*5–10*). Furthermore, T cells also have the capacity to proliferate under T lymphopoienic conditions, and for naïve T cells this too requires recognition of self MHC antigens (*8*, *11–13*). However, less is known about the TCR signals that govern these processes. The src family protein tyrosine kinase p56^{lck} (lck) is involved in the most proximal phosphorylation events during TCR signaling and plays crucial roles at multiple points in T cell development (*14*, *15*). It seemed likely, therefore, that lck would play a critical role in the transduction of survival and homeostatic signals through the TCR.

To evaluate the role of lck in T cell homeostasis, we produced mice that express lck in

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an inducible manner. This was achieved by generating mice on an endogenous lck knockout background, containing transgenes for the reverse tetracycline transactivator (rtTA-C) under the control of human CD2-regulatory elements, ensuring T cell–specific expression. A second lck transgene (Lck1) was coexpressed in these mice under the control of the tetracycline response element (*16*). Lck transgene expression could thus be specifically controlled by the administration of the tetracycline-derivative doxycycline (dox), which was required for transcriptional activity of the rtTA. We have previously reported that induction of transgene expression successfully overcomes the lack of endogenous lck and restores normal thymopoiesis (*16*). The periphery of dox-fed Lck1/rtTA-C/lck^{neg} mice is populated with nearly normal T cell numbers, although there is an increase in the ratio of CD4$^+$ to CD8$^+$ T cells relative to that in wild-type (WT) mice (*17*).

In the first series of experiments, Lck1/rtTA-C/lck^{neg} mice were fed dox (1 mg/ml in drinking water) from before birth, allowing population of the peripheral T cell pool. Between 3 and 6 weeks of age, cohorts of mice were withdrawn from dox, and the fate of peripheral T cells was monitored in the absence of continued Lck1 transgene expression, as compared with littermate controls maintained on dox. The success of this approach, however, was dependent on the complete cessation of lck expression after withdrawal of dox. Analysis of thymic cellularity revealed a rapid atrophy after removal of dox, caused largely by a loss of double-positive (DP) thymocytes. By day 7, thymi from these mice were comparable to those of age-matched lck^{neg} controls (Fig. 1A). Thymic atrophy was shortly preceded by the down-regulation of lck expression, and by day 7 no lck protein was detectable in the thymus (Fig. 1B). To confirm the functional absence of lck, we compared responses to TCR ligation by peripheral T cells from Lck1/rtTA-C/lck^{neg} mice, withdrawn from dox for 2 weeks, with those of lck^{neg} and WT mice. Although frequencies of T cells observed in lymph nodes of Lck1/rtTA-C/lck^{neg} mice were unchanged at this time (Fig. 1C), proliferation and CD69 up-regulation in response to CD3 ligation were severely compromised, resembling those of T cells from lck^{neg} mice (Fig. 1D) (*18*). However, responses of these T cells were restored by addition of dox to in vitro cultures, which reinduced lck expression.

Having determined that lck transgene expression was completely switched off after removal of dox from the diets of adult Lck1/rtTA-C/lck^{neg} mice (hereafter referred to as Lck1 OFF mice), we compared the fate of peripheral T cells in these mice with that of littermates maintained on dox (Lck1 ON mice) as well as aged-matched WT B10 controls. Although expression of lck was essential for full activation of T cells in response to TCR signals (Fig. 1D), continued expression of lck was not required for their prolonged survival (Fig. 2A). Both CD4$^+$ and CD8$^+$ T cells were readily detectable in peripheral blood at time points throughout the experiment and in lymph nodes and spleen of Lck1 OFF mice more than 9 weeks after withdrawal of dox (Fig. 2A). Some decline was observed in the absolute numbers of T cells recovered at the end of the experiment, particularly in the spleen. This is in part due to the functional thymectomy that results in Lck1 OFF mice after abrogation of Lck expression (Fig. 1A). However, analysis of CD44 expression revealed that the frequency of CD44$^{-/low}$ naïve T cells, whose survival is critically dependent on an MHC-derived TCR signal, was largely unchanged during the course of the experiment and between groups. The half-life of naïve T cells in the absence of MHC-dependent TCR signals has been estimated to be less than 3 weeks (*10*), and T cells transferred into hosts lacking appropriate MHC ligands were shown to be completely lost by 2 to 7 weeks (*19*, *20*).

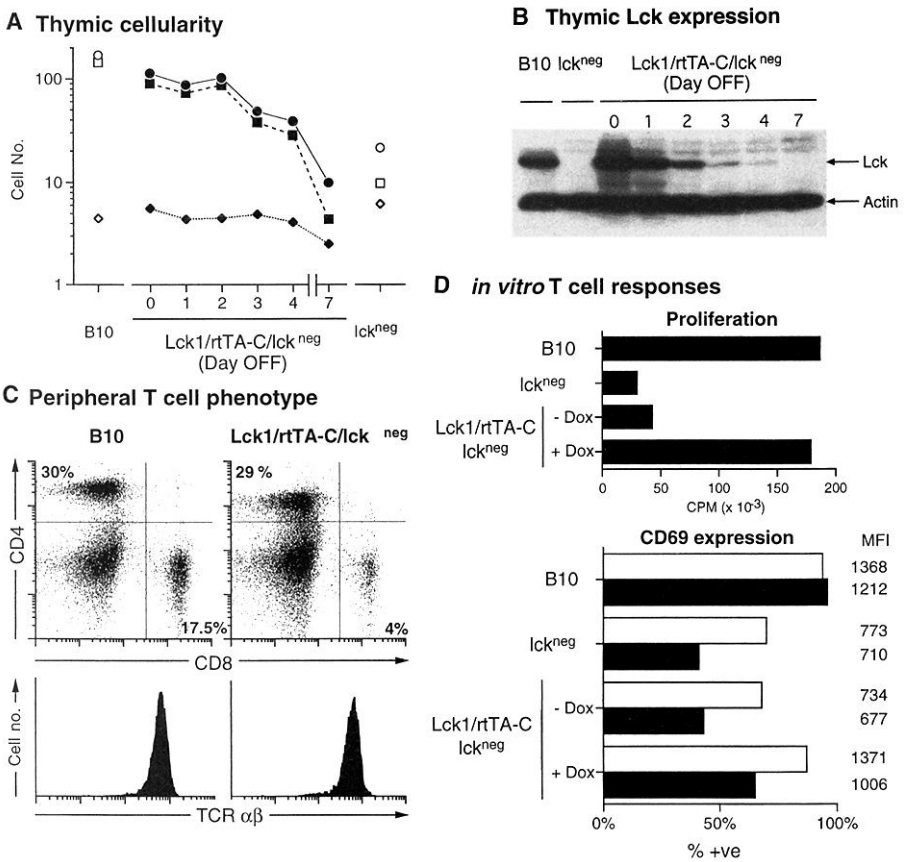

Fig. 1. Abrogation of lck function and protein expression after cessation of dox feeding to Lck1/rtTA-C/lck^{neg} mice. **(A)** Lck1^{het}/rtTA-C^{het}/lck^{neg} mice were bred by crossing Lck1^{hom}/lck^{neg} mice with rtTA-C^{hom}/lck^{neg} mice. Pregnant females were maintained on dox in drinking water (1 mg/ml, sucrose 0.4%), and offspring were fed dox in drinking water after weaning. At 6 weeks of age, Lck1/rtTA-C/lck^{neg} offspring were removed from dox, and thymic cellularity was determined at days 0, 1, 2, 3, 4, and 7 after dox withdrawal (circles, total cell number; squares, DP cell number; diamonds, DN cell number). **(B)** Cell lysates of thymocytes from the mice in (A) were separated on 10% SDS/polyacrylamide gel electrophoresis (5 × 10^6 cells per lane), blotted onto Immobilon-P membrane, and probed for lck protein and actin as control with specific rabbit antisera essentially as described (*16*). **(C)** The phenotype of lymph node cells from Lck1/rtTA-C/lck^{neg} mice 14 days after withdrawal of dox and of age-matched B10 WT controls was determined by staining cells for CD4, CD8, and TCRαβ expression and analyzing on a FacsCalibre (FACS, Becton Dickinson). CD4 versus CD8 dot plots were generated with a live cell gate on the basis of forward and side scatter, and TCRαβ histograms are for a combined CD4$^+$ and CD8$^+$ cell gate. **(D)** The capacity of peripheral T cells to respond to CD3 ligation was determined by purifying T cells from lymph nodes and culturing cells in vitro (10^6/ml) with anti-CD3 monoclonal antibody (mAb) (2C11, 1 μg/ml) and irradiated B10 splenocytes as accessory cells. Proliferation was determined by incorporation of [^{3}H]thymidine added to cultures (0.5 μCi per well) at 42 hours for 6 hours. CD69 expression was determined at 24 hours by staining cultures with CD4-, CD8-, and CD69-specific mAbs and analyzing cells by FACS. The percentage of CD4$^+$ (open bars) or CD8$^+$ (filled bars) blasts staining positive for CD69 expression is shown, and numbers next to bars indicate the mean fluorescence intensity (MFI) of positive cells. Data are representative of three or more experiments.

T cells in Lck1 OFF mice were next examined to confirm that they were still receiving survival signals in the absence of lck expression. Expression of the anti-apoptotic factor Bcl-2 is reduced or lost in naïve T cells not receiving sufficient survival signals (*6*, *21*). Intracellular staining showed equivalent expression of Bcl-2 by T cells from Lck1 OFF mice withdrawn from dox for 9 weeks, and by T cells from Lck1 ON mice and age-matched wild-type controls (Fig. 2C). In further experiments, the CD3ζ chain phosphorylation state was determined. In resting naïve T cells, immunoprecipitation of the TCR complex indicates that the ζ chain of the CD3 complex is constitutively phosphorylated, revealed as a p21 band on a phosphotyrosine blot. It has been suggested that this represents a state indicative of survival signal transduction. Significantly, ζ-chain phosphorylation is lost within 2 weeks of MHC deprivation in vivo (*10*). Analyses of T cells from Lck1 OFF mice at 9 weeks after dox withdrawal by immunoprecipitation of cell lysates with CD3ε antibody showed ζ-chain phosphorylation to be identical to that of T cells from Lck1 ON mice and WT controls (Fig. 2D). These data strongly indicate that lck is not required for maintenance of CD3ζ-chain phosphorylation in resting T cells. Finally, we confirmed total loss of lck in Lck1 OFF mice by Western blot analysis of thymus and peripheral T cell lysates and by the absence of Lck1 transgene mRNA transcripts in peripheral T cells by reverse transcriptase–polymerase chain reaction (RT-PCR) (Fig. 2E).

Potentially, the reduced numbers of T cells in lymphoid organs of Lck1 OFF mice (Fig. 2A) could have resulted from cessation of thymic output. Under normal circumstances, this would be compensated for by expansion of the T cell pool (*22*). We next examined whether naïve T cells failed to undergo homeostatic expansion in the absence of lck. Peripheral T cells were purified from dox-fed Lck1/rtTA-C/lck^{neg} donors, labeled with the cell dye carboxyfluorescein diacetate succinimidyl ester (CFSE), and transferred either into lck^{neg} recipients fed dox, thereby maintaining lck expression in donor T cells, or into dox-free lck^{neg} recipient mice. B10 lymph node T cells were similarly labeled with CFSE and transferred into B10 recipients as controls. To confirm that T cells from Lck1/rtTA-C/lck^{neg} donors could indeed survive in the absence of Lck1 transgene expression, we monitored frequencies of CFSE-positive T cells in the blood of recipient mice over the next 6 weeks. No difference was observed in the decay rate of T cells from dox-fed and dox-free recipients, and CFSE-positive T cells were still readily detectable 6 weeks after transfer (Fig. 3, A and B). Specific survival of naïve cells was confirmed first by monitoring CD44 expression, which was unchanged relative to the donor cell expression before transfer (*23*),

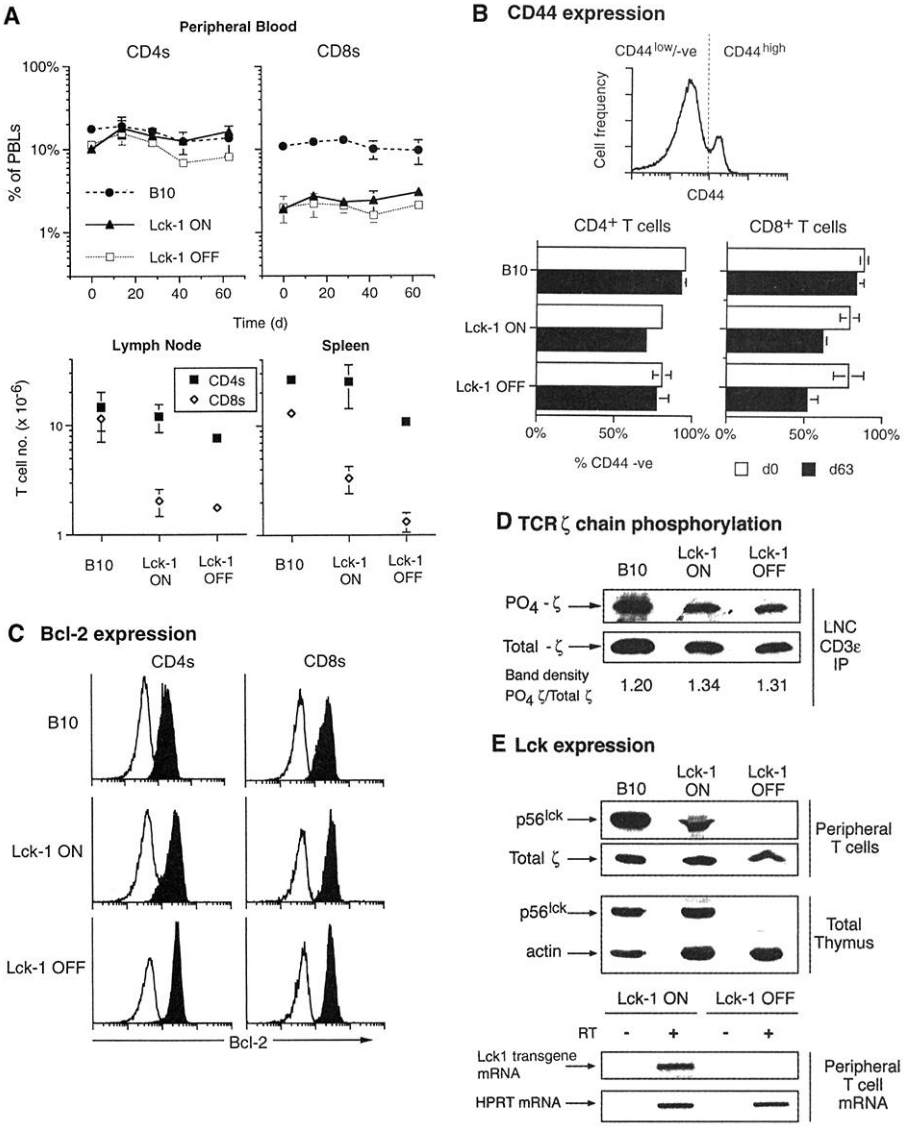

Fig. 2. Prolonged survival of T cells after abrogation of lck expression. (**A**) Groups of 6-week-old Lck1/rtTA-C/lck^{neg} mice, fed dox from conception, were either withdrawn from dox (Lck1 OFF, n = 9) (□) or maintained on dox drinking water as control (Lck1 ON, n = 7) (▲). These mice and age-matched B10 WT controls (n = 4) (●) were bled weekly, and the frequency of CD4$^+$ and CD8$^+$ T cells was determined by staining cells for CD4, CD8, TCRαβ, and CD44 expression and analyzing by FACS. At the end point of the experiment, defined lymph nodes (superficial cervical, brachial, and inguinal) and spleen were removed from mice, and mean numbers of CD4$^+$ (■) and CD8$^+$ (◇) cells were determined for lymph node (lower left) and spleen (lower right). (**B**) CD44 expression by CD4$^+$ and CD8$^+$ peripheral blood T cells from different groups of mice was determined at day 0 after dox withdrawal (open bars) and at the end point of the experiment (d63, filled bars) by FACS analysis. The histogram of CD44 expression by B10 CD8$^+$ T cells indicates the gates used to define CD44$^{-/low}$ and CD44^{high} subsets. (**C**) Bcl-2 expression by CD44$^{-/low}$ lymph node T cells of Lck1 ON, Lck1 OFF, or B10 mice was determined at the experimental end point. Cells were surface-labeled for CD4, CD8, and CD44 expression; fixed (3% paraformaldehyde for 1 hour); and stained with either Bcl-2–specific mAb or isotype control in 0.03% saponin–phosphate-buffered saline. (**D**) TCRζ chain phosphorylation was determined by lysing 10^8 lymph node and spleen T cells from Lck1 ON, Lck1 OFF, and B10 mice at the end point (d63). Lysates were immunoprecipitated with CD3ε-coupled protein A–Sepharose beads overnight. Precipitates were separated on 12.5% SDS–PAGE, blotted onto Immobilon-P membrane, and sequentially probed for phosphotyrosine and CD3ζ as described (*31*). (Upper panel) p21 PO_4 CD3ζ bands; (lower panel) total CD3ζ. Ratio of PO_4 CD3ζ: Total CD3ζ was determined by band density analysis with NIH Image V1.6 software. (**E**) Loss of Lck expression in Lck1 OFF mice removed from dox for more than 10 weeks was confirmed by analyzing thymus and splenic T cell lysates (5×10^6 T cells per lane) by Western blot as described in Fig. 1B. RNA was extracted from purified splenic T cells, and expression of Lck1 transgene mRNA transcripts was determined by RT-PCR (*32*) in the presence or absence of reverse transcriptase (RT) as indicated (bottom panels). Data are representative of three independent experiments.

with most cells remaining CD44$^{-/low}$. Second, CFSE-labeled single-positive (SP) thymocytes from dox-fed Lck1/rtTA-C/lck^{neg} and WT mice exhibited an almost identical pattern of survival, regardless of whether hosts were fed dox or not (Fig. 3, C and D). These cells also remained CD44$^{-/low}$ by the end point (*23*).

WT T cells transferred into "full" syngeneic B10 recipients do not divide (*23*), whereas those transferred into congenitally T cell–deficient lck^{neg} recipients underwent several divisions during this 6 week period (Fig. 4, left columns). T cells from dox-fed Lck1/rtTA-C/lck^{neg} mice transferred into lck^{neg} hosts maintained on dox also underwent several divisions (Fig 4, middle columns), although not as many as WT cells (*24*). Analysis of CD44 expression by dividing CD4$^+$ T cells confirmed that proliferation was occurring among the naïve CD44$^{-/low}$ population for both B10 and Lck1/rtTA-C/lck^{neg}–derived T cells (Fig. 4A). CD4$^+$ T cells from the same Lck1/rtTA-C/lck^{neg} donors transferred into lck^{neg} hosts on a dox-free diet failed to undergo any homeostatic proliferation (Fig. 4A, right column), indicating that sustained lck expression is required for the homeostatic proliferation observed in dox-fed recipients. Examination of the CD8$^+$ compartment revealed similar results. In contrast to mice maintained on dox (Fig. 4B, middle column), CD8$^+$ T cells from the same Lck1/rtTA-C/lck^{neg} donors transferred to water-fed recipients failed to proliferate in response to the T lymphopoenic environment (Fig. 4B, right column). The cycling CD8$^+$ T cells from dox-fed recipients were largely CD44^{high}, unlike the CD4$^+$ cells, and it is not possible to determine whether these cells were derived from CD44^{high} precursors or whether they up-regulated CD44 expression as a consequence of proliferation. Whichever is the case, their expansion is strictly lck-dependent.

Our present findings show that Lck expression is not required for prolonged survival of naïve peripheral T cells. Recent studies examining the MHC-peptide ligands that promote T

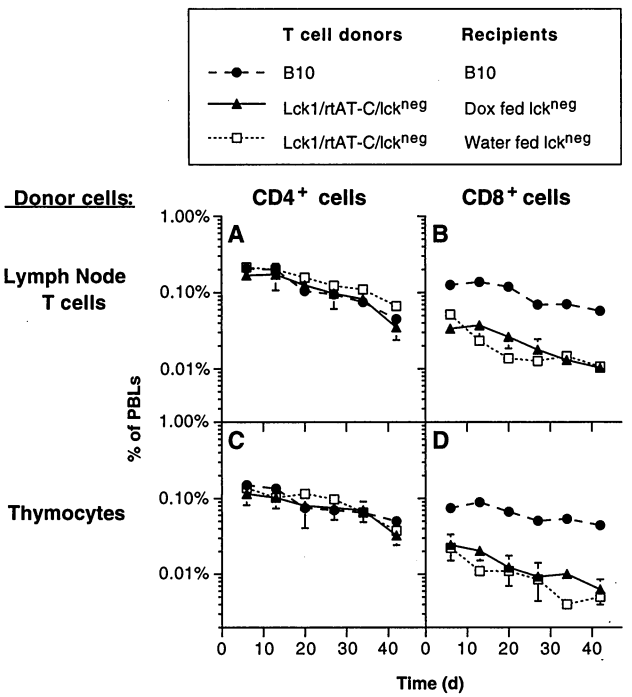

Fig. 3. Survival of T cells from Lck1/rtTA-C/lck^{neg} mice upon adoptive transfer in the absence of continued lck expression. (**A** and **B**) Lymph node T cells from Dox-fed Lck1/rtTA-C/lck^{neg} mice were labeled with CFSE and transferred (5×10^6 cells per recipient) into groups of lck^{neg} recipients either administered dox in their drinking water (▲) or maintained on water alone (□). Lymph node T cells from B10 mice were similarly labeled with CFSE and transferred into B10 recipients as controls (●). Recipient mice were bled weekly, and peripheral blood was stained for expression of CD4 and CD8 and analyzed by FACS (>2×10^5 events per sample) to determine the frequency of CFSE-positive CD4$^+$ (A) and CD8$^+$ (B) T cells present. (**C** and **D**) Thymocytes from the same donor Lck1/rtTA-C/lck^{neg} mice were also labeled with CFSE and transferred (10^8 cells per recipient) into groups of dox-fed lck^{neg} (▲) or water-fed lck^{neg} recipient mice (□). B10 thymocytes were labeled with CFSE and transferred into B10 recipients (10^8 cells per recipient) as control (●). The blood of recipient mice was analyzed to determine the frequency of CFSE-positive SP CD4 (C) and SP CD8 (D) T cells present. Results are the mean ± SD of groups of four mice and are representative of three independent experiments.

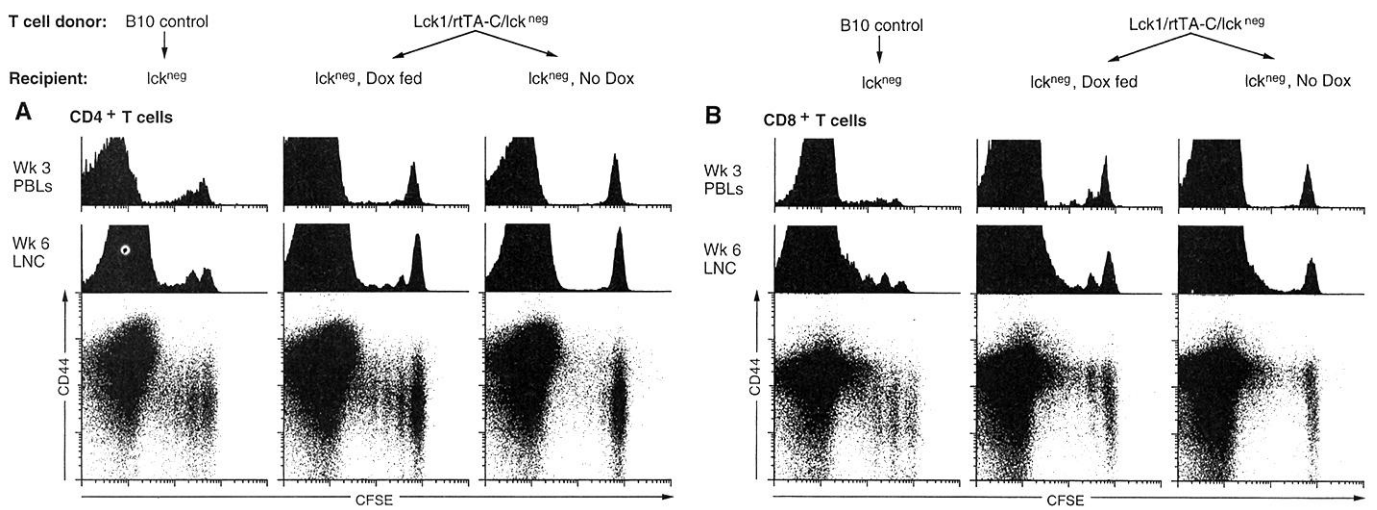

Fig. 4. T cells from Lck1/rtTA-C/lck^{neg} mice require continued lck expression in order to undergo homeostatic proliferation in T lymphopoenic lck^{neg} hosts. Homeostatic proliferation of CD4$^+$ (**A**) and CD8$^+$ (**B**) T cells was determined with the same mice as described in Fig. 3A and an additional group of lck^{neg} mice injected with CFSE-labeled T cells from B10 mice. B10-derived T cells injected into B10 recipients did not undergo any cell divisions during the period of analysis, and all data shown are from CFSE-labeled T cells transferred into lck^{neg} recipient mice. CFSE profiles of transferred B10 T cells (left columns), Lck1/rtTA-C/lck^{neg} T cells transferred into dox-fed recipients (middle columns), and the same cells transferred into water-fed recipients (right columns) were examined in peripheral blood at 3 weeks and by analysis of lymph node cells (LNC) at 6 weeks after transfer by staining for CD4, CD8, and CD44 expression. Dot plots of CD44 expression (*y* axis) versus CFSE label (*x* axis) are of lymph node at 6 weeks after transfer and electronically gated on either live CD4$^+$ (A) or live CD8$^+$ cells (B). Data are representative of three independent experiments.

cell survival and homeostatic proliferation have suggested that low-affinity or antagonist peptides are responsible (*11–13*). It is intriguing, therefore, that homeostatic proliferation of T cells is more lck-sensitive than T cell survival. Although it has previously been assumed that the same signals mediate survival and homeostasis, the data from this study suggest otherwise. We found that T cells survived without lck expression, but that homeostatic proliferation of naïve T cells in T cell–deficient hosts occurred only when lck transgene expression was maintained. This suggests that different signals are required for survival and proliferation, perhaps requiring distinct ligands or different growth and survival factors.

In conclusion, our data suggest that a hierarchy of signals govern T cell behavior. Activation of T cells in response to strong agonist signals is highly lck-dependent, as indicated by the poor T cell responses to CD3 stimulation in the absence of lck (Fig. 2D) and the phenotype of lck-deficient mice (*15*). Homeostatic proliferation may be driven by weak or partial agonist signals that continue to depend on lck activity but that differ from strong agonist signals in that they do not necessarily result in progression of cells to the memory pool (*25, 26*). In contrast, T cell survival signals can be provided by antagonist signals alone that are relatively lck-insensitive (*16, 27*) and may instead depend on other src family members such as fyn (*28, 29*). Although all of these signals may promote survival, only those that activate lck mediate proliferative responses.

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17. That lck is implicated in lineage commitment decisions in the thymus has been demonstrated by a number of recent studies (*16, 30*). In Lck1/rtTA-C/lck^{neg} mice fed dox, levels of the transgene exceed those found in WT mice, conditions favoring the development of CD4$^+$ over CD8$^+$ T cells. Consequently, the periphery of these mice contains fewer CD8$^+$ T cells than the periphery of WT mice, but both CD4$^+$ and CD8$^+$ cells were shown to be of the appropriate MHC restriction (*16*).
18. Both the mean fluorescence intensity and frequency of CD69$^+$ T cells are reduced in the absence of lck.
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24. Whereas intrathymic lck expression in dox-fed Lck1/rtTA-C/lck^{neg} mice is higher than in WT mice, expression by peripheral T cells is considerably lower than in the WT (Fig. 2E), which likely explains the reduced capacity of these cells to undergo homeostatic expansion as compared with WT cells. However, Lck is expressed at functional levels in these mice, as demonstrated by their capacity to undergo homeostatic expansion in vivo.
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32. Splenic T cells were purified from mice by means of mouse T cell–enrichment columns (R&D Systems) and RNA extracted with RNAzol B (Tel-Test). Lck1 transgene transcripts were detected by RT-PCR with primers that specifically amplify a 461–base pair product spanning the mouse Lck and SV40 polyadenylation signal of the transgene construct: forward primer, CAGTACCAGCCCCAGCCTTGATAG (mouse Lck); reverse primer, GCAGTCCAGCTTTTTCCTTTGTGG [SV40 poly(A)]. PCRs were performed in the presence and absence of RT to exclude genomic DNA contamination of RNA, and PCR for HPRT (hypoxanthine-guanine phosphoribosyltransferase) mRNA was performed to ensure that equivalent amounts of total RNA were compared.

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Molecular and Neuronal Substrate for the Selective Attenuation of Anxiety

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Benzodiazepine tranquilizers are used in the treatment of anxiety disorders. To identify the molecular and neuronal target mediating the anxiolytic action of benzodiazepines, we generated and analyzed two mouse lines in which the α2 or α3 $GABA_A$ (γ-aminobutyric acid type A) receptors, respectively, were rendered insensitive to diazepam by a knock-in point mutation. The anxiolytic action of diazepam was absent in mice with the α2(H101R) point mutation but present in mice with the α3(H126R) point mutation. These findings indicate that the anxiolytic effect of benzodiazepine drugs is mediated by α2 $GABA_A$ receptors, which are largely expressed in the limbic system, but not by α3 $GABA_A$ receptors, which predominate in the reticular activating system.

Excessive or inappropriate anxiety can be controlled by enhancing inhibitory synaptic neurotransmission mediated by GABA (GABAergic inhibitory neurotransmission) using clinically effective benzodiazepine drugs (*1*). However, to date it has not been possible to identify the one or more $GABA_A$ receptor subtypes that mediate the attenuation of anxiety. Four types of diazepam-sensitive $GABA_A$ receptors can be distinguished on the basis of the presence of α1, α2, α3, or α5 subunits. These receptors can be rendered insensitive to diazepam in vitro by replacing a conserved histidine residue by arginine in the drug binding site (*2, 3*). Introduction of the respective point mutation into mouse lines enables the pharmacological profile of benzodiazepine drugs to be attributed to defined receptor subtypes. Using this approach, we have attributed the sedative and amnesic prop-

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