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The conversion of mature somatic cells into pluripotent stem cells, both by nuclear transfer and transduction with specific "reprogramming" genes, represents a major advance in regenerative medicine. Pluripotent stem cell lines can now be generated from an individual's own cells, facilitating the generation of immunologically acceptable stem cell-based therapeutics. Many cell types can undergo nuclear reprogramming, leading to the question of whether the identity of the reprogrammed cell of origin has a biological consequence. Peripheral blood, containing a mixture of T, B, NK, and myeloid cell types, represents one potential source of reprogrammable cells. In this study, we describe the unique case of mice derived from a reprogrammed T cell. These mice have prerearranged T-cell receptor (TCR) genes in all cells. Surprisingly, ≈50% of mice with prerearranged TCR genes develop spontaneous T cell lymphomas, which originate in the thymus. The lymphomas arise from developing T cells, and contain activated Notch1, similar to most human and mouse T-cell acute lymphoblastic lymphomas. Furthermore, lymphomagenesis requires the expression of both prerearranged TCRα and TCRβ genes, indicating a critical role for TCR signaling. Furthermore, inhibitors of multiple branches of TCR signaling suppress lymphoma growth, implicating TCR signaling as an essential component in lymphoma proliferation. The lymphomagenesis in mice derived from a reprogrammed T cell demonstrates the deleterious consequences of misregulation of the TCR rearrangement and signaling pathways and illustrates one case of cellular reprogramming where the identity of the cell of origin has profound consequences.

induced pluripotent stem cells | lymphoma | notch | receptor-mediated leukemogenesis | T cell receptor

Advances in nuclear reprogramming of somatic cells have led to novel methods of creating pluripotent stem cell lines and represent an important step toward the generation of patient-specific, stem cell-based therapies. Reprogramming to pluripotency has been achieved by transfer of a somatic cell nucleus into an enucleated oocyte, and by enforcing the expression of specific reprogramming genes in somatic cells (1, 2). These two methods have been used to reprogram multiple different cell types, including fibroblasts, liver cells, pancreatic β cells, neural progenitor cells, and T and B cells (2–5). Although many cell types can undergo nuclear reprogramming, the underlying differences between pluripotent stem cell lines derived from distinct somatic cell types remain largely undetermined.

We have described a line of mice, LN3, which was derived from a reprogrammed lymph node T-cell nucleus (6). In LN3 mice, every somatic cell contains the recombined T-cell receptor (TCR) genes of the original LN3 donor T cell, whereas normally TCR gene rearrangement occurs only in T cells during a specific stage in their development. We showed that T-cell development in LN3 mice was altered because of the accelerated expression of the germ-line LN3 TCRs. Here, we show that LN3 mice harboring functionally recombined TCR genes undergo T-cell lymphomagenesis.

T-cell acute lymphoblastic lymphomas and leukemias (T-ALL) represent 10–25% of acute lymphoblastic lymphomas and leukemia (ALL) cases in humans and arise from developing T cells within the thymus (7). Several genetic abnormalities have been implicated in the pathogenesis of T-ALL, including transcription factor genes in the basic helix–loop–helix, Lim domain containing, and homeobox families, and genes involved in TCR and cytokine signaling (7, 8). Among the most predominant genetic lesions are activating mutations within Notch1, which occur in >50% of cases (9). Notch also plays an essential role in normal thymocyte development, where it drives both proliferation and differentiation of early progenitors, indicating that overlapping signaling pathways drive both normal and malignant proliferation of T-cell progenitors.

The TCR signaling pathway may represent another important component of lymphomagenesis. The invariant preTCRα gene (which acts as a surrogate for TCRα during T-cell development) has been shown to cooperate with Notch signaling to induce lymphomagenesis in mice (10). More directly, mice transgenic for components of the TCR have been shown to undergo lymphomagenesis, including mice that overexpress a TCRα transgene, mice that overexpress CD3ε, and mice that express truncated versions of TCRβ (11–13). These findings suggest that the TCR signaling pathway can play a role in driving lymphomagenesis and also bring up the question of whether a completely unmodified TCR expressed at normal levels can be oncogenic.

The LN3 mice were originally generated through the transfer of the nucleus of a mature T cell into an enucleated egg; this reprogrammed cell was used to generate an embryonic stem cell line and, subsequently, the original LN3 mice. The resultant mice were backcrossed multiple generations to the C57BL/6 strain, with each generation selected to maintain the productive TCRα and TCRβ rearrangements (2, 6). The antigen specificity of the prerearranged TCR is unknown, but the LN3 receptor is MHCI restricted (6). We showed that the prerearranged TCR genes were expressed earlier than their unrearranged counterparts, resulting in abnormal T-cell development, which was defined by accumulation in the thymus of a $CD4^{-}CD8^{-}CD3^{+}$ population and by a hypertrophic thymic environment (6). During the course of those experiments, we noticed that many of the LN3 mice developed T-cell lymphomas, implicating the prerecombined TCR genes as potential oncogenes and demonstrating a unique phenotype associated with pluripotent cell lines derived from reprogrammed T cells.

Results

Prerearranged TCR Genes Drive Lymphomagenesis. The lymphomas that develop in LN3 mice are characterized by an enlarged

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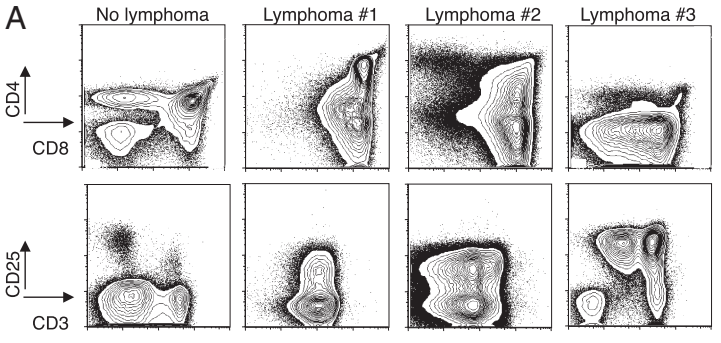
thymic mass composed of blasting cells, which are also often present in the spleen, lymph nodes, and bone marrow. The surface phenotypes of the lymphomas are variable, and each lymphoma is often composed of several phenotypically distinct subpopulations that resemble sequential stages of thymocyte development, based on expression of CD4, CD8, and CD25 (Fig. 1*A*). Each lymphoma subpopulation also expresses surface CD3, indicating the presence of a surface TCR (because the surface expression of CD3 and TCR subunits are concomitant) and consistent with the fact that these cells contain prerearranged TCR genes (Fig. 1*A*).

Strikingly, we observed that lymphomas only occurred in mice that carried both the prerearranged TCRα and TCRβ genes (LN3) and that no lymphomas developed in mice that expressed only one (LN3α) or the other (LN3β) (Fig. 1*B*). TCRα and TCRβ must be coexpressed and pair to form a viable TCR; therefore, the correlation of their coexpression with lymphomagenesis suggested that expression and signaling of a fully assembled TCR drove the generation and/or growth of the lymphomas. The lymphomagenesis also correlated with the timing of full-length TCR expression, which began abnormally early during T-cell development in LN3αβ mice (Fig. S1 and ref. 6). In addition, the ability of these two prerearranged and coexpressed TCR genes to drive lymphomagenesis was maintained in mice multiple generations removed from the original cloned mice. Because epigenetic abnormalities in cloned animals are generally repaired upon passage of DNA through the germ line, we conclude that the prerearranged TCR genes, rather than epigenetic abnormalities, drive lymphomagenesis (14–16).

To investigate the role of canonical TCR signaling in the lymphomagenesis of LN3 mice, we generated LN3 mice that lacked both β2M and I-A^b, and therefore lacked classical MHCI/peptide and MHCII/peptide complexes. In these mice, T-cell development is largely blocked at the DP stage, consistent with the requirement of MHCI for positive selection of LN3 thymocytes. Interestingly, lymphomas still developed in these mice at a similar rate to the mice that expressed both MHCI and MHCII, indicating that the development of lymphomas in the LN3 mice required the mature TCR, but did not require the TCR to interact with classical MHCI or MHCII molecules (Fig. 1*B*). It remains unclear whether signaling through the TCR of the LN3 mice drives lymphomagenesis through a noncanonical ligand or a ligand-independent mechanism. In addition, RAG expression was not required for lymphomagenesis in LN3 mice, because LN3 mice crossed to the $RAG2^{-/-}$ background also developed lymphomas (Fig. 1*B*). However, in all mice analyzed, regardless of RAG or MHC deficiencies, the coexpression of both the prerearranged TCRα and TCRβ chains was required for lymphomagenesis.

Notch Signaling Is Active in Lymphomas of LN3 Mice. The unusual origin of these lymphomas led us to investigate the pathways that drive their transformation and proliferation. Notch activation plays a critical role in T-cell commitment and development and is a hallmark of T-ALL (9). In T-cell progenitors, Notch signaling is initiated by binding to delta-like ligands expressed on thymic epithelial cells, leading to proteolytic cleavage and nuclear transport of the transcription activating, intracellular domain of Notch (17). In lymphomas, Notch can become constitutively activated by mutations that facilitate its proteolytic activation or by mutations that increase the stability of the cleaved, activated form (18). The activated form of Notch1 can be detected by an antibody that recognizes its free cleaved end, enabling the determination of Notch1 activation within cell populations (19). To investigate whether this pathway is activated in the LN3 lymphomas, we performed Western blots on cell lysates of several primary lymphomas by using an antibody specific for the cleaved, activated form of Notch1. We found that 15 of 17 primary lymphomas had activated Notch1 as detected by Western blot, including lymphomas that lacked either RAG or MHC molecules. Twelve of the lymphomas expressed an activated form of Notch1 which migrated at the expected molecular weight of the activated, wild type protein, but 3 lymphomas expressed truncated forms of activated Notch1, showing that LN3 lymphomas, like human T-ALL, aberrantly activate Notch1 genes through multiple molecular mechanisms. These results indicate that activation of the Notch pathway represents one of the common hits that drive lymphomagenesis in LN3 mice.

Activation of TCR-Signaling Components Drives Lymphoma Growth. In addition to Notch activation, lymphomagenesis in LN3 mice requires functional prerearranged TCR genes, leading us to investigate the importance of TCR signaling in lymphoma proliferation. One major downstream transcriptional effector of TCR activation is the nuclear factor of activated T cells (NFAT), which translocates to the nucleus after stimulation of the TCR, and is essential for both T-cell development and T-cell activation (20). To determine whether NFAT is localized to the nucleus in



B

TCR genotype	Lymphoma incidence (7 months)	
Wt	0% (0/15)	
LN3	46% (6/13)	p=0.004
LN3 α–only	0% (0/15)	
LN3 β–only	0% (0/11)	p=0.01
LN3 $\beta2M^{-/-}$	64% (9/14)	p=0.02
LN3 $\beta2M^{-/-}$ $IA^{b-/-}$	43% (3/7)	
LN3 $RAG^{-/-}$	25% (4/16)	

Fig. 1. LN3 lymphomas are located in lymphoid organs and have an immature thymocyte phenotype. (*A*) Surface phenotype of nonleukemic (*Left*) and three separate leukemic thymuses stained for molecules normally associated with T-cell development, indicating that the individual lymphomas have heterogeneous phenotypes that partially overlap with several stages of immature thymocyte development. (*B*) Offspring from a cross of wt × LN3 mice were analyzed over a 7-mo period for onset of lymphoma. Mice that showed signs of illness were euthanized, and lymphomas were verified by dissection as greatly enlarged thymuses, spleens and/or lymph nodes. Lymphomas were verified by flow cytometry. LN3 mice contain both prerearranged TCRα and TCRβ; LN3α contain only the prerearranged TCRα; LN3β contain only the prerearranged TCRβ; wt indicates littermates that lacked prerearranged TCR loci; LN3 $\beta2m^{-/-}$ mice do not express surface MHCI molecules; LN3 I-$A^{b-/-}$ mice lack MHCII molecules; and LN3 $RAG^{-/-}$ mice are RAG2-deficient. In all crosses studied, only mice that expressed both the prerearranged TCRα and TCRβ chains developed lymphomas. Statistical *P* values were calculated by using Fisher's exact test.

LN3 lymphomas, we transduced an LN3 lymphoma cell line that had adapted to culture with an NFAT-eGFP fusion protein that reproduces the localization of the native protein (21). In these cells, we observed a stable and low level of NFAT-eGFP fluorescence both in the nucleus and the cytoplasm, evident as a diffuse green staining throughout the cells (Fig. 2*B*). This partial nuclear localization of NFAT-eGFP in untreated cells contrasts to the state of the cells upon acute activation of the TCR-signaling pathway by incubation with the pharmacological agents, phorbol myristate acetate (PMA), and ionomycin (22). Rapidly after PMA/ionomycin stimulation, the NFAT-eGFP becomes almost entirely localized to the nucleus of the lymphoma cells, and is absent from the periphery and near the plasma membrane (Fig. 2*B*). In contrast, treatment of the lymphoma cells with the calcineurin inhibitor, cyclosporin A (20), caused the rapid exclusion of NFAT from the nucleus, revealing that LN3 lymphomas have chronically activated the NFAT-signaling pathway, possibly through TCR activation (Fig. 2*B*).

To determine whether NFAT signaling was biologically important in lymphoma cell growth, we measured the effect of cyclosporin A on the proliferation of four independent LN3 lymphoma lines. We found that low concentrations of cyclosporin A inhibited growth of each lymphoma line over a 3-d culture period (Fig. 3*B*). The susceptibility of the lymphomas to cyclosporin A indicated that NFAT activation plays an important role in the proliferation of LN3 lymphomas and suggested that signaling molecules upstream of NFAT, such as the TCR, might be mediating lymphoma proliferation.

Inhibitors of TCR and Notch Signaling Block Lymphoma Growth. To test whether more proximal mediators of TCR signaling were also activated in the LN3 lymphomas, we tested the sensitivity of the cells to inhibitors of two kinases that are associated with the TCR synapse, LCK and PKCθ. LCK is associated with CD4, and LCK-mediated phosphorylation of the ITAMs of the TCR-associated, CD3 complex is an essential, and very rapid, event downstream of TCR engagement (23). In parallel, PKCθ is an essential component of TCR-mediated T-cell activation and colocalizes with the TCR synapse (24). We found that inhibitors of both of these kinases specifically suppressed LN3 lymphoma growth, whereas they had no effect on the growth of a B-cell hybridoma line (Fig. 3 *C* and *D*). The fact that both of these kinases are required for proliferation of the LN3 lymphomas strongly implicates TCR activation as a critical component of LN3 lymphoma proliferation. Given the parallels between the LN3 lymphomas and human T-ALL, these data suggest that inhibitors of NFAT activation, such as cyclosporin A, as well as novel inhibitors of PKCθ, such as CG0471, which is used here, might also effectively inhibit the growth of human T-ALL. Notably, cyclosporin A has been shown to induce apoptosis of human T-ALL cells in culture (25, 26).

Because we had observed that the Notch pathway was active in these lymphomas, we tested the ability of the lymphomas to grow in the presence of an inhibitor of gamma secretase, which is the activating protease of Notch (27). We found that this inhibitor suppress growth of all of the lymphoma lines tested and also had no effect on the growth of a B-cell hybridoma at two different concentrations (Fig. 3*A*). Notch activation in LN3 lymphomas is reminiscent of human T-ALL, for which constitutive activation of Notch is also a common trait (9). Notably, the cell line that was least sensitive to the gamma secretase inhibitor (RAGLN3 #64) was derived from a primary lymphoma that did not show activated Notch by Western blot (Figs. 2*A* and 3*A*). This same cell line was also the most sensitive to inhibition of the TCR signaling pathway, suggesting that the TCR signaling pathway can act independently of Notch to drive lymphoma growth.

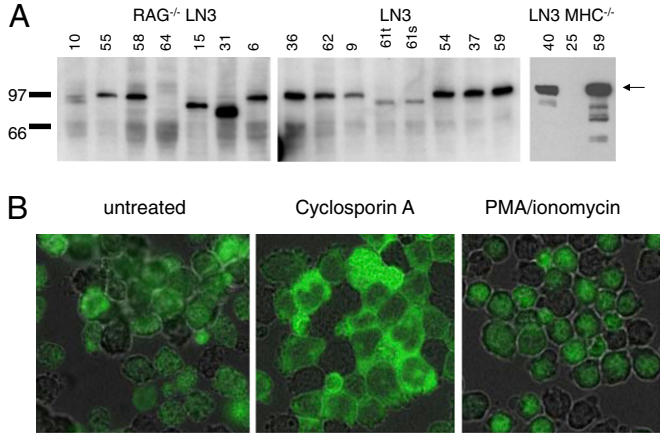


Fig. 2. LN3 lymphomas have activated Notch and NFAT signaling pathways. (*A*) Western blots were performed on cell lysates from 17 individual LN3 lymphomas, using an antibody that recognizes the activated (cleaved) form of Notch. Fifteen of 17 LN3 lymphomas stained positive for the cleaved form of Notch1 (indicated by arrow), showing that this pathway is activated in the majority of lymphomas derived from LN3 mice, regardless of RAG expression ($RAG^{-/-}$) or β2M and IA^b expression ($MHC^{-/-}$). (*B*) NFAT-eGFP is partially nuclear localized in untreated lymphoma cells. Two independent LN3αβ lymphoma-derived cell lines (LN3A and LN3B) were transduced with a retrovirus encoding an NFAT-eGFP fusion protein. Representative data are shown from one of these lines (LN3B). Cells were either left untreated, or treated in culture for 30 min with PMA (1 μM) + ionomycin (250 ng/mL) or cyclosporin A (10 μM), and then plated onto glass slides with coverslips and immediately analyzed for on a fluorescence microscope. Untreated cells have a partially nuclear localized NFAT-eGFP, as compared to cells that have almost entirely nuclear NFAT-eGFP (PMA/ionomycin treated) and cells that have almost entirely cytoplasmic NFAT-eGFP (cyclosporin A treated).

Discussion

Implications for Induced Pluripotent Stem (IPS) Cells. Although many different cell types can be reprogrammed into pluripotent cell lines, the biological properties of reprogrammed cells that are determined by the cell type of origin have remained unclear. In this study, we show that mice that are several generations removed from a clone derived from a T-cell nucleus retain the tendency to undergo lymphomagenesis. This lymphomagenesis required specific prerearranged TCRα and TCRβ genes, indicating that the tendency toward lymphomagenesis was due to the T-cell origin of the original clone and, specifically, the expression of prerearranged TCR genes. It is unclear at this point whether lymphomagenesis is a general phenomenon of mice with prerearranged TCR genes, but given that TCR transgenic mice can also develop spontaneous lymphomas, TCR-mediated lymphomagenesis may be common in mice with misregulated TCR expression (11). Current methods of reprogramming often begin with cellular sources such as cord blood, bone marrow, and peripheral blood: all tissues that contain complex mixtures of cell types, including T cells. This study suggests that precautions should be taken to ensure that the identity of the reprogrammed cell of origin is known, and that T cells, and probably also B cells, are not inadvertently turned into therapeutic IPS cells. Recent studies have used human blood-derived T cells as sources of IPS cells, and these cells promise to be valuable tools for studying human immune development and disease; however, the results presented here indicate that extra caution is warranted regarding the therapeutic use of such T cell-derived IPS cells (28–30).

Implications for Gene Therapy. Transfer of tumor-specific TCR genes into hematopoietic stem and progenitor cells has been used to establish antitumor immunity in mice (31). Our observations in the LN3 mice suggest that this approach might predispose the transduced progenitors toward lymphomas. Furthermore, our previous analysis of LN3 mice showed that developing T cells that express the prerearranged TCR genes developed very inefficiently

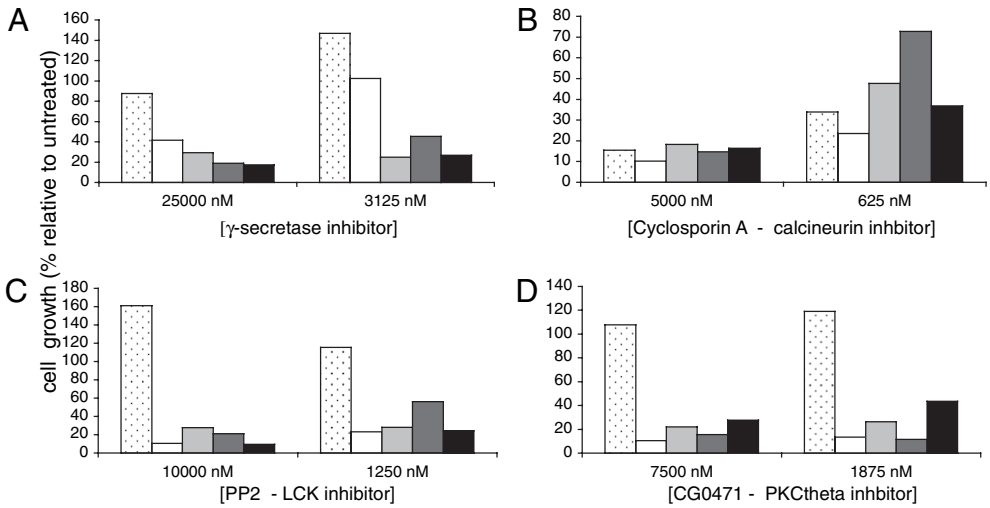


Fig. 3. Inhibitors of Notch1 activation, NFAT signaling, LCK, and PKCθ all block LN3 lymphoma proliferation. Four independent LN3 lymphoma cell lines: RAGLN3 #64 (white bars), RAGLN3 #31 (light gray bars), LN3 A (dark gray bars), LN3 B (black bars), and one B cell hybridoma (dotted bars, as a control for nonspecific toxicity) were plated at 5,000 cells per well and were treated with a γ-secretase inhibitor (*A*), cyclosporin A (*B*), an Lck inhibitor (*C*), or a PKCθ inhibitor (*D*). Three days later, live cells were counted by flow cytometry, and percent growth was calculated as: 100 × (number of cells in treated well) / (number of cells in untreated wells). Results are representative of at least four independent experiments for each inhibitor and each cell line. A titration of inhibitors were used in each assay, and concentrations that did not affect B cell hybridoma growth were chosen where possible to ensure that the inhibitors were not acting through nonspecific toxicity (this control for toxicity was not possible for cyclosporin A, which is known to also inhibit B-cell lymphoma growth).

in the presence of wild-type cells (6). This aberrant and lymphomagenic development of LN3 thymocytes likely stems from the early expression of the TCR. These observations suggest that future approaches that use TCR gene transduction into hematopoietic stem cells will benefit from using promoters that drive TCR expression at more appropriate (later) time points in development.

Implications for Lymphomagenesis. The occurrence of lymphomas in the LN3 mice is reminiscent of the lymphomagenesis that has been reported in HY TCR transgenic mice (11). In those mice, it was possible that the lymphomagenesis was due to a transgenic insertion site that activated an oncogene; however, in the LN3 strain, the prerearranged TCR genes are in the normal locus and, therefore, these results definitively show that prerearranged TCR genes can drive lymphomagenesis.

The mechanism that enables prerearranged TCR genes to drive lymphomagenesis is unclear. The lymphoma proliferation requires activation of TCR signaling components, including LCK, PKCθ, and NFATc1, implying that either an activating mutation has occurred within the TCR-signaling pathway or that the TCR is being ligated by an unidentified factor. It remains possible that constitutive activation of the TCR signaling pathway spontaneously arose during the culture adaptation of the lines used in this study. Although this possibility has not been excluded here, the essential role of the prerearranged TCR in the initiation of the LN3 lymphomas in vivo implicates TCR signaling in the origin of the lymphomas. The involvement of cell surface mitogenic receptors such as the TCR in leukemogenesis has precedent: Moloney virus-induced thymic lymphomas in BALB/c and other susceptible strains can express cell-surface receptors specific for the inducing retrovirus, creating a mitogenic loop (32–35). In that model, thymic T-cell clones that bind intact retrovirus self-select for infection and mitogenic expansion; the expanding cells could derive subclones that activate other protooncogenic events and/or inhibit tumor suppressors (36). During natural development of thymic lymphomas in the highly lymphoma-susceptible AKR mouse, the viruses that emerge to bind the lymphomas are always recombinant between integrated proviruses in the envelope (env) region, presenting new "antigenic" surfaces to the host (33, 37). In the AKR mouse, between 25 and 50 wk of life, the virus-binding subpopulation expands; in a preview of the cancer stem cell hypothesis (38), only the virus-binding T cells transfer the lymphoma to syngeneic mice, whereas their coexisting nonbinding T cells are nonleukemogenic (33, 37). In one such case, the binding of a leukemogenic retrovirus to its cognate leukemia cell line could be inhibited by antibodies to either CD4 or TCR, implicating the TCR as the leukemogenic receptor (39). Similarly, virus-binding surface Ig can play a mitogenic role in viral-mediated B cell leukemogenesis in mice and humans (40, 41). Also, in Friend leukemia virus-induced erythroleukemias, the retroviral env product appears to bind the erythropoietin receptor on these cells (42).

In the case of the LN3 mice, the TCR is required for both initiation and ongoing proliferation of lymphomas. Interestingly, this TCR-mediated lymphomagenesis occurs even in the absence of a classical ligand, because MHCI$^{-/-}$ II$^{-/-}$ LN3 mice undergo lymphomagenesis at the same rates as the MHC-expressing LN3 mice. The TCR-dependent, MHC-independent lymphomagenesis of LN3 mice suggests that either a nonclassical ligand drives the LN3 lymphoma to proliferate or, alternatively, that the TCR is chronically activated in the absence of ligation, possibly by proximal activating mutations in the signaling pathway. A viral-mediated transformation that activates the TCR signaling pathway also remains possible.

Targeting the TCR signaling pathway might be a productive strategy for inhibiting growth of lymphomas, because cyclosporin A has been shown to kill human lymphoma cells in vitro and murine lymphoma cells in vivo (25, 26). Previous studies of mice transgenic for activated Notch showed that signaling through the pre-TCR is necessary for lymphomagenesis (43). Similarly, the present study shows that misregulated TCR signaling alone can be a first step in leukemogenesis and such lymphomas use the TCR signaling pathway for proliferation.

Methods

Mice. The LN3 mice have been described (6). They originated from the nuclear transfer of a lymph node T cell from a 129/SvJae × C57BL/6 F_1 male mouse into an enucleated oocyte. The original clones were bred more than six generations to the C57BL/6 background, with genotyping to maintain the pre-

rearranged TCR genes. RAG2$^{-/-}$, β2M$^{-/-}$, and I-A$^{b-/-}$ mice, all on the C57BL/6 background, were originally obtained from JAX Labs (Bar Harbor, ME). Mice were housed in the Stanford University Medical Center, and the Stanford Animal Use and Care Committee approved all experiments involving animals.

Cell Lines and Culture. Four LN3 lymphoma cell lines were derived from independent mice. These lines were derived by disaggregation of lymphomic thymuses into single cells, and culture of cells in IMDM + 10% FCS in the presence of 10 ng/mL interleukin 7. These cultures typically slowly died off, and then rare cells proliferated and gave rise to immortalized cell lines, which were IL-7 independent. An antibody-producing B cell hybridoma, PC.61, was used as a non-T-cell lymphoma control for the inhibitor experiments. Inhibitors of γ-secretase (γ-secretase IX), Lck (PP2), and NFAT (cyclosporin A) were purchased from Calbiochem. The PKCθ specific inhibitor, CG0471, was identified from a chemical library at Complegen and has an IC_{50} of 0.1 μM for PKCθ, 1.2 μM for PKCδ, 0.9 μM for PKCε, and 0.1 μM for PKCη.

NFAT-eGFP and Microscopy. Two independent LN3 lymphoma lines were infected with a retrovirus encoding NFAT-eGFP (a generous gift of Andrew T. Girvin). Transduced GFP$^+$ cells were sorted by FACS and cultured for further experiments. Cells were treated with phorbol 12-myristate 13-acetate (250 ng/mL) and ionomycin (1 μM), or with cyclosporin A (10 μM), or left untreated for 30 min at 37 °C, and were then immediately placed on slides and were analyzed on a Nikon Eclipse E800 microscope equipped with a mercury lamp and a fluorescent filter for GFP.

Antibodies, Flow Cytometry, and Cell Sorting. Flow cytometry and cell sorting was performed either on a FACSaria equipped with 405-, 488-, and 633-nm lasers or on a highly modified Benton Dickinson FACS machine by using argon 405-, 488-, and 598-nm lasers, and data were collected by using DIVA digital electronics. Analysis of data was performed with FlowJo (TreeStar). Live cells were gated based on forward and side scatter and propidium iodide exclusion. Antibodies to CD3ε (KT31.1), CD4 (GK1.4), CD8 (53-6.7), and CD25 (PC61) were either purchased directly conjugated to phycoerythrin (PE), Cy7PE, or Cy5PE (BD Pharmingen) or were purified from hybridoma supernatants and directly conjugated to fluorochromes Pacific blue, Pacific orange, Alexafluor 488, or Alexafluor 647 (Molecular Probes).

Western Blots. Cells were lysed in 1% Triton X-100 in PBS on ice in the presence of protease inhibitor mixture III (Calbiochem). Lysates were cleared by centrifugation at 14,000 × *g* for 15 min, and the equivalent of 100,000 cells were separated on an 8% SDS/PAGE gel and blotted onto PVDF (Amersham). Blots were blocked with 10% BSA fraction V, or with 5% nonfat milk, in 0.05% Tween 20, 150 mM NaCl, 20 mM Tris at pH 7.5, and then rabbit antibody to activated Notch 1 (Cell Signaling) was added at a final concentration of 1:2,000. Blots were washed extensively and then incubated with 1:5,000 dilution of goat anti-rabbit horseradish peroxidase (Invitrogen). Blots were developed by using a bioluminescent substrate (Amersham), and light-sensitive film (Kodak).

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