Thymic Expression of a T Cell Receptor Targeting a Tumor Associated Antigen Co-Expressed in the Thymus Induces T-ALL

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RUNNING HEAD: Survivin Reactive TCR Induces T-ALL

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Key Points:

- Thymocyte signaling via a transgenic survivin reactive TCR induced T-ALL with 100% penetrance.

- Thymic expression of signaling receptors targeting tumor-associated antigens co-expressed in the thymus poses a risk for leukemogenesis.
Abstract

T cell receptors and chimeric antigen receptors recognizing tumor-associated antigens can now be engineered to be expressed on a wide array of immune effectors. Engineered receptors targeting tumor-associated antigens have most commonly been expressed on mature T cells, however some have postulated that receptor expression on immune progenitors could yield T cells with enhanced potency. We generated mice (Sur-TCR-Tg) expressing a TCR recognizing the immunodominant epitope (Sur_{20-28}) of murine survivin during early stages of thymopoiesis. Spontaneous T cell acute lymphoblastic leukemia (T-ALL) occurred in 100% of Sur-TCR-Tg mice derived from three separate founders. The leukemias expressed the Sur-TCR and signaled in response to the Sur_{20-28} peptide. In preleukemic mice, we observed increased cycling of double negative thymocytes expressing the Sur-TCR and increased nuclear translocation of NFAT, consistent with TCR signaling induced by survivin expression in the murine thymus. β2M^{−/−} Sur-TCR-Tg mice, which cannot effectively present survivin peptides on Class I MHC, had significantly diminished rates of leukemia. We conclude that TCR signaling during the early stages of thymopoiesis mediates an oncogenic signal, and therefore expression of signaling receptors on developing thymocytes with specificity for tumor-associated antigens expressed in the thymus could pose a risk for neoplasia, independent of insertional mutagenesis.
**Introduction**

Adoptive immunotherapy has shown increasing promise as a treatment for cancer, driven in part by advances in genetic engineering that permit efficient expression of receptors targeting tumor antigens on immune effectors. Several clinical trials have demonstrated antitumor efficacy following adoptive transfer of mature T cells engineered to express T cell receptors (TCRs) targeting tumor-associated antigens (TAA), including NY-ESO-1, MART-1 and MAGE-A. Similarly, impressive antitumor effects have recently been observed following transfer of mature T cells engineered using retroviruses to express chimeric antigen receptors targeting tumor-associated antigens. In these studies, toxicity has related to autoimmunity and cytokine release syndrome, but oncogenesis as a result of retroviral based gene transfer has not been observed.

This stands in contrast to the clinical experience following gene therapy for IL2Rγc congenital immunodeficiency where five of twenty patients developed T-ALL following retroviral mediated expression of IL2Rγc in hematopoietic stem cells. Leukemia in this setting was associated with insertional mutagenesis, with integration of the IL2Rγc transgene into regulatory regions of the LMO2 oncogene, leading to dysregulated LMO2 expression. Although insertional mutagenesis mediated by the retroviral vector was an essential component of leukemogenesis observed in this series, retroviral vectors encoding the adenosine deaminase transgene have integrated into regulatory regions of oncogenes, including LM02 and MDS1-EVI1, following gene therapy for correction of adenosine deaminase deficiency, without the development of leukemia. One possible explanation for these findings is a contribution of IL2Rγc signaling in early thymocytes to leukemogenesis, a hypothesis that remains
controversial. TCR signaling during early thymopoiesis appears sufficient to induce T-ALL in some animal models, and in others the aberrant TCR signal plays a cooperating role as part of an oncogenic cascade. Interestingly, T-cell ALL induced in patients receiving IL2Rγc expressing hematopoietic progenitors was associated with other oncogenic mutations, in particular those involving the *NOTCH1* oncogene. Thus, while insertional mutagenesis poses one risk for gene therapy involving hematopoietic progenitors, expression of signaling receptors themselves during early thymopoiesis may also be oncogenic, especially when combined with cooperating mutations in partner oncogenes.

Because extensive T cell differentiation is associated with diminished functionality and diminished persistence following adoptive transfer, there has been increasing interest in expressing receptors engineered to recognize tumor-associated antigens in less differentiated T cell progenitors, including multipotential hematopoietic progenitors. In this study, we created transgenic mice expressing a TCR recognizing a peptide derived from survivin, a tumor-associated molecule expressed in some non-neoplastic tissues, including the thymus, that has been studied as a potential target for immunotherapy of cancer. The Sur-TCR was expressed downstream of a human CD2 promoter, which drives expression during early stages of thymopoiesis. Sur-TCR-Tg T cells seeded the periphery of Sur-TCR-Tg mice, but did not mediate autoimmunity or meaningful antitumor effects. However we unexpectedly observed T cell acute lymphoblastic leukemia (T-ALL) in 100% of Sur-TCR-Tg mice derived from three separate founders. All Sur-TCR-Tg associated T-ALLs also had NOTCH1 mutations. Through a series of studies, we demonstrate that signaling via the Sur-TCR, in response
to MHC restricted presentation of peptides within the thymus, plays an essential oncogenic role in this model system. This model demonstrates that genetic engineering aimed at endowing T lymphoid progenitors with the capacity to recognize tumor-associated antigens, which are co-expressed in the thymus, could pose a risk for neoplasia, independent of insertional mutagenesis.

MATERIALS AND METHODS

Generation of Survivin TCR Transgenic Mice

Animal studies were conducted according to NCI Animal Care and Use Committee approved protocols. Wild-type (WT) C57BL/6 mice were purchased from the Animal Production Unit, National Cancer Institute (NCI; Frederick, MD). Rag1−/− and β2M−/− mice were purchased from the Jackson Laboratory. To generate Sur-TCR-Tg mice, C57BL/6 mice were vaccinated twice one week apart with 100 μl of a Db-binding peptide derived from mouse survivin20-28 (Sur20-28) plus a CD4+ helper peptide (HBVc128-140) in incomplete Freund’s adjuvant. Splenocytes were harvested 2-wk after vaccination and cultured with Sur20-28 peptide (0.1 ug/ml) pulsed irradiated EL4 cells (18,000 rads) with 10IU/ml rhIL-2 for 14 days. Sur20-28 tetramer binding CD8+ splenocytes were electronically sorted, then RNA was extracted and TCR alpha- and beta-chains were cloned using 5’-Rapid Amplification of cDNA Ends (5’-RACE, Life Technologies). TCR α and β transcripts were found to be Vα8/Jα24/Cα and Vβ13/Dβ2/Jβ2/Cβ2. The α and β genomic sequences were PCR amplified with primers gα1 (5’-AGCTGAATTCATGAACAGATTCCTGGG-3’) and gα2 (5’-AGCTGTCGACTCAACTGGACCACAGCC-3’) or gβ1 (5’-
AGCTGAATTCAATGGGCTCCAGGC -3’) and gβ2 (5’-
AGCTGTCGACGTCAAGGATGCATAA-3’), respectively. Sequence validated purified PCR products were cloned into a hCD2 minigene based vector as previously described31 (kindly provided by Al Singer NCI, NIH), and co-injected into fertilized C57BL/6 embryos yielding eleven transgenic founder lines. Three founder lines with a high frequency of Sur20-28 tetramer binding CD8+ T cells were bred, housed at the National Institutes of Health (NIH) and underwent further analysis.

**Immunofluorescence and Imaging Flow Cytometry**

To assess NFAT nuclear localization using immunofluorescence, two independent Sur-TCR-Tg+ leukemic cell lines were infected with a retrovirus encoding NFAT-eGFP (a generous gift of Irving L. Weissman, Stanford University). GFP+ cells were sorted by flow cytometry, then treated with Sur20-28 (2 µg/ml), or phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 µM) or untreated for 30 min at 37°C, then cytospun onto slides (800rpm for 3min). Cells were fixed (4% paraformaldehyde) for 30min at 4°C on slides. Propidium iodide (PI) was used for nuclear staining before mounting with VECTASHIELD (Vector laboratories). Nuclear translocation was assessed as positive or negative via visual assessment using a Nikon Eclipse E800 microscope with a mercury lamp and a fluorescent filter. Images were captured with a Zeiss AxioCam MRm digital camera; quantitative analysis was performed using Zeiss Zen 2011 software.

Cells stained for imaging flow cytometry were run on an ImageStreamX using INSPIRE software (Amnis Corporation, Seattle, WA). A Brightfield area lower limit was set so debris and particles with an area of ≤ 25 pixels were not saved. A Raw Max
Pixel (non-background subtracted pixel value) upper limit was set to channel 4094 for all fluorescence channels so any pixels saturating the camera were not saved into the data file. A minimum of 20,000 cells meeting these acquisition criteria were acquired per sample at 40X magnification. Single color compensation controls were acquired with the Brightfield illumination and the 785 laser turned off. A compensation matrix was created and all analyses were performed in IDEAS analysis software (Amnis Corporation, Seattle, WA). Single cells were gated using a Brightfield Area versus Brightfield Aspect Ratio plot and by drawing a gate around the single cell cluster having a high aspect ratio. Cells having nuclei in focus were gated using the Gradient RMS feature on the Dapi channel (channel 7). All further analyses were performed on single cells with nuclei which were in focus.

To assess NFAT nuclear translocation, thymocytes from 6-week old Sur-TCR-Tg+ (black) and WT (grey) mice were stained with cell surface antibodies (CD4, CD8, CD25, CD44, Ter119, CD11b, CD11c, Gr1), fixed/permeabilized (eBioscience) overnight at 4°C, stained with intracellular NFATc2 (G1-D10, Santa Cruz) for 30min, then AF488 goat-anti-mouse IgG2a (Invitrogen) antibody (1:400) for 30 min. Dapi was added before analysis. Colocalization of NFAT and Dapi was measured using the Similarity feature, a pixel-by-pixel comparison of the two images (IDEAS software, Amnis).

Results

*Mature Peripheral T Cells In Sur-TCR-Tg Mice Bind Survivin Peptides, But Do Not Respond To Naturally Processed Survivin Peptides On Tumors Or Normal Tissues*
To explore the potential for survivin reactive TCRs to mediate antitumor effects, we generated TCR transgenic mice expressing a TCR with specificity for an H-2b restricted peptide in murine survivin (Sur\textsubscript{20-28}). Based upon binding of > 90% of CD8\textsuperscript{+} T cells to tetramers incorporating Sur\textsubscript{20-28}, three Sur-TCR-Tg transgenic founders (E8, F8 and L8) were bred for further studies (Fig. 1A). Sur-TCR-Tg T cells, but not WT cells, specifically proliferated (Sup Fig. 1A) and degranulated (Fig. 1B), as evidenced by surface expression of CD107a\textsuperscript{a},\textsuperscript{32} in response to Sur\textsubscript{20-28}. Because survivin is expressed at low levels on a variety of normal tissues\textsuperscript{33}, which could activate Sur-TCR-Tg T cells, we assessed whether peripheral T cells in Sur-TCR-Tg mice showed evidence of T cell activation. There was no evidence for aberrant T cell activation based upon CD44 expression on BV8\textsuperscript{+} T cells in WT mice versus Sur-TCR-Tg mice (Figure 1C), and >99.5% of T cells in Sur-TCR-Tg\textsuperscript{+/Rag}\textsuperscript{1\textsuperscript{-}} mice, which do not express endogenous alpha chains bore a naïve phenotype (Fig. 1C). Further, sur-TCR-Tg T cells did not produce cytokines when co-cultured with H-2b\textsuperscript{+}Sur\textsuperscript{+} tumors \textit{ex vivo} (Sup. Fig. 1B, 1C), or mediate antitumor effects when transferred into H-2b\textsuperscript{+}Sur\textsuperscript{+}tumor bearing recipients \textit{in vivo} (Sup. Fig. 1D). Since the thymus expresses survivin at levels equivalent to tumor cell lines (Sup. Fig. 1B), we reasoned that aberrant thymic development could result in maturation of non-functional Sur-TCR-Tg cells. To test this, we tested functionality of retrovirally transduced healthy C57Bl/6 splenocytes expressing the Sur-TCR, which confirmed responsiveness to Sur\textsubscript{20-28}, but an absence of response to Sur-expressing tumor cell lines (Sup. Fig. 2). Together, the data are consistent with a model wherein mature Sur-TCR-Tg T cells are capable of responding to Sur\textsubscript{20-28}, but do not recognize naturally processed survivin peptides expressed on normal or malignant tissues likely related to
insufficient signal strength as a result of low TCR avidity and/or inefficient peptide processing or presentation\textsuperscript{34-36}.

**Leukemic Transformation Occurs In Developing Thymocytes In All Sur-TCR-Tg Mice From Three Separate Founders**

Unexpectedly, Sur-TCR-Tg\textsuperscript{+} mice from all three founders spontaneously developed T cell acute lymphoblastic leukemia (T-ALL). Death due to leukemia began at 4 months of age in F8 and L8 offspring and at 6 months of age in E8 offspring (Fig. 2A). Incidence and timing of onset of the leukemia was identical in Sur-TCR-Tg/Rag\textsuperscript{+/−} vs Sur-TCR-Tg/Rag\textsuperscript{−/−} mice from each founder (Fig. 2A), and all animals succumbed to leukemia by 14 months of age. The longer leukemic latency for progeny of the E8 founder corresponded with a lower site density and frequency of Sur-TCR-Tg\textsuperscript{+} T cells compared to the other two founders (Fig 1A), raised the prospect that the Sur-TCR might contribute to leukemogenesis.

T-ALL in Sur-TCR-Tg mice is characterized by enlargement of the spleen, thymus, lymph nodes, lung, liver and kidney due to infiltration with sheets of TdT\textsuperscript{+} blasts (Fig. 2B, 2C, Sup. Fig. 3), which are typically CD3\textsuperscript{+}CD24\textsuperscript{−}TRBV8\textsuperscript{+}CD25\textsuperscript{−}CD4\textsuperscript{−}CD8\textsuperscript{−} (Fig. 3A). Sur-TCR-Tg T-ALL is transplantable, since leukemic cells from enlarged thymi and spleens injected intravenously into sub-lethally irradiated (500 rad) WT recipients resulted in lethal T-ALL within 3 weeks in 8/8 recipients. Further, 10 of 10 unirradiated recipients of cells from an established Sur-TCR-Tg T-ALL cell line developed T-ALL, with a mean life expectancy of 30 days.
To test whether T-ALL in Sur-TCR-Tg mice bore a functional Sur-TCR, we transduced T-ALL cells with an NFAT reporter construct expressing GFP, then exposed them to the Sur\textsubscript{20-28} peptide. Fluorescence microscopy demonstrated significantly increased nuclear NFAT translocation following exposure of T-ALL cells to the Sur\textsubscript{20-28} peptide, consistent with intact signaling via the transgenic Sur-TCR (Fig. 3B). Together the data are consistent with T-ALL derived from neoplastic transformation of a thymocyte expressing the transgenic Sur-TCR.

To explore whether leukemogenesis in this model derived from a singular transforming event or occurred in multiple cells contemporaneously, we exploited incomplete allelic exclusion of the TCR alpha chain\textsuperscript{37-39}, and analyzed the TRAV (TCR alpha variable) repertoire in Sur-TCR-Tg T-ALL. Fig. 3C demonstrates diverse TRAV gene rearrangement in WT mice, and significant TRAV diversity in Sur-TCR-Tg pre-leukemic mice, studied at 6-8 weeks of age, prior to the onset of T-ALL. Interestingly, the TRAV repertoire of cells derived from effaced spleens from mice with T-ALL demonstrated gene rearrangement involving the majority of TRAV families, consistent with leukemia-transforming events involving multiple thymocytes contemporaneously. Alternatively, it is possible that TRAV gene rearrangement occurred subsequent to transformation. In contrast, cell lines derived from \textit{ex vivo} passage of T-ALL cells harvested from affected organs were clonal, demonstrating only one native alpha rearrangement, (TRAV12 in the representative line shown in Fig. 3C), as well as TRAV8 (derived from the transgenic alpha chain). We conclude that transgenic expression of the Sur-TCR gives rise to highly penetrant leukemic transformation, observed in three
separate founders, which are transplantable, and may occur contemporaneously in multiple thymocytes.

T-ALL In Sur-TCR-Tg Mice Does Not Require Oncogene Activation Via Insertional Mutagenesis

Since leukemia occurred in three independent Sur-TCR-Tg founders, with similar latencies and penetrance, we judged it statistically highly unlikely to be caused by insertional mutagenesis. Nonetheless we sought to map insertion sites using linker-mediated PCR of genomic DNA. A restriction enzyme resistant band representing the site of vector-genomic DNA junction ascertained in splenocyte DNA extracted from a progeny of founder E8 mapped the insert site to a gene desert on chromosome 14 (Sup. Fig. 4A), 351kb upstream (5’) of dachshund homolog 1 (Dach1), and 516kb downstream (3’) of mitotic-spindle organizing protein 1 (MZTI). The insertion site was confirmed by shotgun sequencing, however PCR based amplification of the same E8 vector/insertion junction was not successful in progeny of F8 and L8 founders, confirming that integration did not occur in the same site in these animals. Expression of Dach1 did not differ in E8 progeny compared to WT mice and Mzt1 expression was unchanged in the brain and modestly downregulated in the thymi in E8 progeny when compared to WT mice (Sup. Fig. 5).

Linker mediated PCR was not successful in identifying the insertion sites in progeny of F8 and L8 founders, therefore we used a FISH hybridization probe, which bound the human CD2 enhancer, and identified transgene integration into chromosome 11 for F8 and L8 founders. Interestingly, FISH analysis revealed four copies of
chromosome 11 in leukemia lines derived from F8 and L8 progeny, with two of the four copies of chromosome 11 carrying the transgene, consistent with genomic instability in T-ALL lines derived from a Sur-TCR-Tg°/° heterozygote (Sup. Fig. 4B). Together the data make insertional mutagenesis a highly unlikely mechanism for highly penetrant leukemia in progeny from three founders, further suggesting that the Sur-TCR itself was implicated in leukemic transformation in this model.

**Preleukemic Sur-TCR-Tg Mice Show Increased Cycling and Expansion of Early Thymocyte Subsets**

To further explore the pathogenesis of leukemic transformation in this model, we studied the thymi of Sur-TCR-Tg animals prior to the onset of leukemia. At 6 weeks of age, preleukemic Sur-TCR-Tg thymi were abnormally small. Between 6 weeks and 4 months, WT thymi decreased in size whereas Sur-TCR-Tg thymi increased in size, such that by 4 months of age, Sur-TCR-Tg thymi were of similar size to WT thymi (Fig. 4A). Despite their small size, we observed increased frequencies and absolute numbers of CD4⁻CD8⁻ double negative (DN) thymocytes and CD8⁺CD4⁻ in 6 wk Sur-TCR-Tg thymi, compared to WT mice (Fig. 4B), while CD4⁺CD8⁺ double positive and CD4⁺CD8⁻ thymocytes were reduced compared to WT. The increased numbers of CD8⁺CD4⁻ was due to expansion of immature single positive CD8⁺ cells (ISPs), as they expressed higher levels of CD24 and lower levels of CD127 than CD8⁺ SP thymocytes found in WT mice (Sup. Fig. 6)

Using CD44 and CD25 expression to subclassify the DN subset, we observed that only the CD44⁻CD25⁻ DN4 subset was increased in frequency in preleukemic Sur-TCR-
Tg mice (Fig. 4C), consistent with previous reports in other TCR-Tg mice\textsuperscript{40,41}. To explore the basis for preferential enrichment of the DN4 subset and the CD8\textsuperscript{+} SP subset, we measured BrdU incorporation in thymocytes from preleukemic Sur-TCR-Tg vs age matched WT mice. Sur-TCR-Tg mice showed significantly increased rates of BrdU incorporation in DN2 and DN3 subsets, essentially entirely mediated via cycling of thymocyte progenitors that expressed the Sur-TCR, as evidenced by TRBV8 expression (Fig. 4D). We also observed increased BrdU incorporation in CD8 SP thymocytes, which also expressed the Sur-TCR. Together, the data are consistent with a model wherein Sur-TCR expression leads to excessive cycling in DN2, DN3 and CD8\textsuperscript{+} ISP thymocytes, resulting in expanded populations of DN4 and CD8\textsuperscript{+} ISP thymocytes.

**Signaling via the Sur-TCR Plays a Critical Role in Leukemic Transformation**

Previous work demonstrated that thymi express robust levels of survivin\textsuperscript{33} and we confirmed that survivin RNA was expressed at similar levels in thymocytes, thymic epithelium and solid tumors (Sup. Fig 1B, Sup Fig. 7). We thus reasoned that Sur-TCR expressing thymocytes were likely exposed to survivin derived peptides presented via H-2b during thymic development, potentially inducing TCR signaling that could contribute to leukemic transformation. To determine whether we could observe evidence for signaling via the Sur-TCR, we utilized ImageStream technology, which simultaneously measures cellular morphology, immunofluorescence, and cell surface phenotype by flow cytometry, in order to measure NFAT nuclear translocation in specific thymocyte subsets. Nuclear translocation of NFAT was used as a surrogate for TCR signaling, and was assessed based upon co-localization with DAPI, generating an NFAT/Dapi similarity
score for each cell analyzed. For illustration, **Figure 5A**, left panel shows immunofluorescence of four representative DN thymocytes with NFAT/Dapi similarity scores of 0.5 (non-translocated), whereas the right panel shows representative DN thymocytes with NFAT/Dapi scores of 3.0 (highly translocated). **Figure 5B** shows the NFAT/Dapi similarity score histograms for gated DN thymocytes from one representative Sur-TCR-Tg (black line) vs. WT mouse (grey line). Mean NFAT/Dapi similarity scores for DN thymocytes in Sur-TCR-Tg mice were significantly higher than scores in WT mice (2.62±0.03 vs. 1.84±0.06 from Sur-TCR-Tg vs WT mice respectively, \( p = 0.0013 \)). Using a value of >1.0 similarity to define cells with NFAT nuclear translocation, we quantified NFAT translocated cells in thymocyte subsets from preleukemic Sur-TCR-Tg vs WT mice at 6 weeks of age (**Fig. 5C, top**). Pre-leukemic Sur-TCR-Tg mice had significantly increased numbers of DN thymocytes with NFAT translocation, whereas NFAT translocation in other thymic subsets was not abnormal. When DN subsets were analyzed, significant increases in NFAT nuclear translocation were present in the DN2 and DN4 subset in Sur-TCR-Tg preleukemic mice compared to WT mice (**Fig. 5C, bottom**). Together these results are consistent with thymic expression of survivin derived peptides inducing signaling via the Sur-TCR, resulting in expansion of early thymocyte subsets. It is important to acknowledge however that signaling via the Sur-TCR could also potentially occur in response to cross-reactive peptides within the thymus.

To address whether Class I presentation of survivin derived peptides was essential for leukemogenesis, we generated Sur-TCR-Tg/β2M\(-\) animals and monitored them for development of T-ALL (**Fig. 5D**). Unlike the Sur-TCR-Tg mice, which had high rates of
leukemia onset beginning at 6 months, we observed diminished leukemia in Sur-TCR-Tg/β2M⁻/⁻ mice \((p=0.0006)\). This data directly implicates presentation of peptides on Class I MHC as an essential element in leukemogenesis observed in Sur-TCR-Tg mice.

**NOTCH1 Mutations in T-ALL in Sur-TCR-Tg mice**

Notch1 activation plays a critical role in thymocyte survival and maturation, and NOTCH1 mutations are a hallmark of T-ALL\(^{42,43}\). We observed Notch1 overexpression in the spleen of Sur-TCR-Tg T-ALL mice (Figure 6A), and all samples studied, including five primary Sur-TCR-Tg tumors and four Sur-TCR-Tg leukemic cell lines, contained Notch1 mutations (Table 1). PEST mutations were most frequent and often accompanied by additional Notch1 mutations (5’deletion or HD mutation), whereas 5’ deletion and HD mutations were mutually exclusive. Notably, no Notch1 mutations were found among 15 animals tested at 6 wk of age, in the pre-leukemic stage. To test whether murine Sur-TCR-Tg leukemic cell lines with Notch1 mutations were sensitive to γ-secretase inhibitor (GSI) treatment, we cultured three independently derived Sur-TCR-Tg leukemic cell lines in the presence of GSI (Figure 6B). The GSI had little effect on L8-3, which has only a 5’ deletion mutation (Table 1) that is not sensitive to gamma secretase inhibition. However GSI significantly suppressed cell growth of F8-3 \((p<0.05\) on Day 6 versus control) and L8-2 leukemic cell lines \((p<0.05\) on Day 5 and 6 versus control), both of which contain PEST domain mutations in combination with either HD mutations or 5’ deletions (Table 1). These studies are similar to results in numerous previously published reports demonstrating the important role for notch1 signaling in maintaining the viability of transformed T-ALL cells\(^{42,43}\).
We also sought to determine whether TCR signaling might contribute to continued viability of Sur-TCR-Tg T-ALL. Treatment of T-ALL cell lines generated from Sur-TCR-Tg mice with CsA led to significant growth inhibition of L8-2, but not L8-3 and F8-3, and we did not observe significant reduction in NFAT nuclear localization following treatment with CsA ($p=0.33$) (Sup. Fig. 8A). To more directly inhibit TCR signaling, we attempted to knockdown CD3ζ via transduction of an shRNA targeting this protein in L8-3 and F8-3. As shown in Supplemental Figure 8B, the approach achieved 40-60% knockdown of CD3ζ RNA, but this resulted in no detectable difference in cell proliferation or apoptosis compared to the lines transduced with control shRNA (Sup. Fig. 8C and 8D). Hence, the results generated here do not implicate a continued role for Sur-TCR signaling in sustaining the T-ALL, with the caveat that the maneuvers undertaken likely did not completely block signaling via the TCR pathway.

**Discussion**

Cancer immunotherapy is experiencing increasing success, in part due to identification of targets that can provide the basis for T cell recognition of cancer. Tumor-specific antigens, such as mutated proteins or idiotypes, are preferred targets for antigen-specific immunotherapy, but their specificity varies greatly across individuals making them difficult to target broadly. Alternatives are tumor-associated antigens, molecules highly expressed in cancer, with limited expression in normal tissues. Among tumor-associated antigen candidates, survivin was ranked highly for potential immunotherapeutic targeting based upon prominent expression in virtually every human cancer, undetectable levels in most normal adult tissues and its likely important role in
oncogenesis. However, a previous report illustrated fratricide of survivin expressing T cells in dense cultures ex vivo as a pitfall of targeting survivin using gene transfer of a high affinity survivin-specific TCR into mature T cells.

In this study, we discovered a second barrier to using gene therapy to target survivin, which may have relevance for targeting of other tumor-associated antigens. Sur-TCR-Tg mice, which express a TCR recognizing murine survivin during early thymopoiesis, unexpectedly developed T-ALL with 100% penetrance. Leukemia occurred with similar incidence and penetrance in offspring of three founders, ruling out insertional mutagenesis and potentially implicating the Sur-TCR itself in providing an oncogenic signal. We demonstrate that early thymocyte progenitors expressing the Sur-TCR undergo enhanced cycling and expansion in vivo associated with NFAT translocation, consistent with TCR signaling, and that animals with defects in presentation of Class I peptides develop leukemia at greatly reduced rates. Given that Class I peptide presentation is not entirely absent from β2m−/− animals, we posit that the data are most consistent with a model wherein TCR signaling during the early stages of thymopoiesis provides an oncogenic hit, which is followed by acquisition of NOTCH1 mutations, and ultimately gives rise to full blown leukemia. The basis for the high mutation rate of NOTCH1 during early thymopoiesis is not well understood, but it is clear that NOTCH1 is the most common oncogene implicated in T-ALL leukemogenesis and can cooperate with multitude oncogenes to induce T-ALL.

Thus, the process of leukemogenesis observed in this model shows similarities with several other animal models of T-ALL, as well as T-ALL observed in patients treated with IL2Rγc based gene therapy. In both this model and the clinical experience
with $IL2R\gamma c$ retroviral based gene therapy, a receptor capable of providing a potent activation stimulus was expressed during the early stages of thymopoiesis and in both cases $NOTCH1$ mutations cooperated to generate full transformation. While the issue of whether $IL2R\gamma c$ expressed during early thymopoiesis can provide a leukemogenic signal is unresolved, several animal models have implicated the TCR as an oncogene in T-ALL$^{23-26}$. In some cases, TCR signaling during early thymopoiesis appears sufficient to induce T-ALL, whereas in others the TCR signals plays a cooperating role as part of an oncogenic cascade. Pre-TCR signaling has also been implicated in T-ALL development as a cooperating event$^{25,50}$. Unlike the $IL2R\gamma c$ scenario however, we could find no evidence for insertional mutagenesis, which would have activated a third oncogene in this model system, although we cannot rule out other cooperating events. While the process was highly efficient, only occurring in 100% of animals observed, essentially all T-ALLs in this model co-expressed $NOTCH1$ mutations leading us to conclude that TCR signaling in this model system is likely to be only one of at least two hits required for the full oncogenic transformation. Indeed, the extent to which these T-ALL remain dependent upon TCR signaling is less clear, since some cell lines showed growth inhibition in response to CsA, whereas other did not. Furthermore, although studies are ongoing, we could not induce regression of T-ALL in animals treated with CsA who were inoculated with Sur-TCR-Tg T-ALL.

Although the thymus and cancer cell lines express similarly high levels of survivin, it remains unclear why peripheral T cells expressing the Sur-TCR did not expand efficiently in response to survivin expressing tumors, whereas the same TCR expressed on early thymocyte progenitors was sufficient to initiate an activation signal
that likely initiated the oncogenic transformation. This could reflect lowered threshold for TCR triggering in developing thymocytes versus mature T cells and/or differential efficiency of antigen presentation by thymic epithelium versus tumors.

Current trials of gene therapy for treatment of cancer have largely focused on engineering mature T cell populations, however several groups have elegantly demonstrated that T cell differentiation and ultimately, T cell exhaustion, are fundamental factors limiting the potency of adoptive T cell therapies\(^{28,51}\). For this reason, some investigators have considered delivering receptors capable of inducing immune cell activation onto progenitors, thus endowing the most stem-like immune populations with tumor specificity\(^{29}\). Such receptors span native T cell receptors, affinity enhanced T cell receptors, or potentially synthetic receptors incorporating TCR zeta with costimulatory endodomains. The data presented here raises a note of caution for such approaches and are consistent with previous work directly demonstrating increased susceptibility of hematopoietic progenitors to leukemogenesis when compared to mature T cells\(^{52}\). One might expect that many tumor-associated antigens could also be expressed in the thymus and/or that cross-reactive antigens could trigger signaling during the early stages of thymopoiesis. Based upon the evidence presented here and several model systems where strong activation signals in developing thymocytes initiate lymphomogenesis\(^{53}\), we propose that such approaches could pose a risk for neoplasia, independent of insertional mutagenesis. Preclinical studies that assess whether specific receptors targeting tumor antigens will be activated during the early stages of thymopoiesis \textit{in vivo} may be important for minimizing risk.
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Authorship Contributions

Yongzhi Cui: Generated and interpreted data, wrote the manuscript
Masahiro Onozawa: generated and interpreted data
Haven Garber: generated and interpreted data
Leigh Samsel: generated and interpreted data
Ziyao Wang: generated and interpreted data
Philip McCoy: guided experimental design
Sandra Burkett: generated and interpreted data
Xiaolin Wu: generated and interpreted data
Peter Aplan2: oversaw data generation, data interpretation
Crystal Mackall1: oversaw data generation, data interpretation, co-wrote the manuscript

Conflict-of-Interest Statements

The authors report no conflicts of interest.
References


Table 1. Notch1 Mutations in T-ALL in Sur-TCR-Tg mice

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<td>WT</td>
<td>66280 G &gt; GTTTTCCCCCT</td>
<td>Neg</td>
</tr>
<tr>
<td>F8-1</td>
<td>T-ALL Thymus</td>
<td>WT</td>
<td>66280 G &gt; CCG</td>
<td>Pos</td>
</tr>
<tr>
<td>F8-2</td>
<td>T-ALL Thymus</td>
<td>WT</td>
<td>66280 G &gt; CC</td>
<td>Pos</td>
</tr>
<tr>
<td>L8-1</td>
<td>T-ALL Thymus</td>
<td>59724 T&gt;C (Leu &gt; Pro)</td>
<td>66280 G &gt; CC</td>
<td>Neg</td>
</tr>
<tr>
<td>F8-3</td>
<td>T-ALL Cell line 1</td>
<td>59730 T&gt;G (Ile &gt; Ser)</td>
<td>66280 G &gt; CCG</td>
<td>Neg</td>
</tr>
<tr>
<td>L8-2</td>
<td>T-ALL Cell line 2</td>
<td>WT</td>
<td>66279 C &gt; AC</td>
<td>Pos</td>
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<tr>
<td>L8-3</td>
<td>T-ALL Cell line 3</td>
<td>WT</td>
<td>WT</td>
<td>Pos</td>
</tr>
<tr>
<td>L8-4</td>
<td>T-ALL Cell line 3</td>
<td>WT</td>
<td>66278 A &gt; ACCCCT</td>
<td>Neg</td>
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HD Domain: heterodimerization domain
Figure Legends

Figure 1. Survivin-specific T cells from Sur-TCR-Tg mice respond to survivin\textsubscript{20-28} peptide. (A) CD8\textsuperscript{+} T cells from PBL of Sur-TCR-Tg mice bind survivin tetramer (gated CD8\textsuperscript{+} splenocytes are shown from E8, F8 and L8 founders and wild-type (WT) mice. (B) Sur-TCR-Tg CD8\textsuperscript{+} splenocytes (left panel) express high levels of CD107a following stimulation by Surv\textsubscript{20-28} (solid line), compared to control E7 peptide (dotted line). Splenocytes from WT mice show no significant degranulation when exposed to the same peptide (right panel). (C) Expression of CD44 and TRBV8 (TCR beta variable) on gated CD8\textsuperscript{+} peripheral blood lymphocytes from WT, Sur-TCR-Tg, and Sur-TCR-Tg/Rag\textsuperscript{-/-} mice. Results are shown from one representative mouse, and similar results were observed in more than 6 animals of each type.

Figure 2. Sur-TCR-Tg mice develop lethal leukemia. (A) Leukemia developed in progeny of all three founders beginning at 4-6 months, with equivalent latency and penetrance in Sur-TCR-Rag\textsuperscript{+/-} and Sur-TCR-Rag\textsuperscript{-/-} mice. (B) T-ALL mice (right) had enlarged spleens (top) and thymi (bottom) at necropsy compared to WT mice (left). (C) Hematoxylin and eosin (H&E) staining of thymus (top), and anti-TdT immunohistochemistry of spleen (bottom) in pre-leukemic 6 week old Sur-TCR-Tg mice (left) and T-ALL 6 month old Sur-TCR-Tg mice (right) (50x magnification). T-ALL mice show complete effacement of the affected organs. (B) and (C) show examples from one representative mouse, and similar results were observed in more than 6 animals of each type.
Figure 3. T-ALL in Sur-TCR-Tg mice is comprised of transformed thymocytes that express and signal via the transgenic TCR. (A) Flow cytometry of splenocytes harvested from representative WT, Sur-TCR-Tg pre-leukemic mice (approx. 6 weeks of age) and Sur-TCR-Tg T-ALL mice. T-ALL cells consistently express CD3, CD24 and TRBV8, usually express CD25 and typically do not express CD4 or CD8. Similar results were observed in at least three separate experiments involving more than 6 animals of each type. (B) Sur-TCR-Tg T-ALL signal in response to the Sur20-28 peptide. T-ALL cell lines derived from a representative Sur-TCR-Tg leukemic mouse were transduced with NFAT-eGFP, then exposed to the irrelevant control E7 peptide, Sur20-28, or PMA/Ca ionophore. Nuclear translocation of NFAT-eGFP as identified via microscopy is shown in a representative field (top) and data is summarized from 10 fields for each condition (bottom). Four independent experiments using two separate T-ALL cell lines were tested with similar results. (C) TRAV gene expression in representative WT and Sur-TCR-Tg pre-leukemic mice reveal a diverse repertoire. T-ALL harvested from spleen of a representative T-ALL mouse expresses an oligoclonal repertoire of TRAV genes, whereas a cell line derived from leukemic splenocytes is clonal, as evidenced by a single native TRAV gene (TRAV12 arrow). Pre-leukemic spleen, leukemic spleen and the T-ALL cell lines, (but not WT) express the transgenic TRAV8, designated with a star. This experiment was repeated at least three times using three different leukemic and WT animals as well as three separate leukemic lines with similar results.

Figure 4. Preleukemic Sur-TCR-Tg mice demonstrate perturbed thymopoiesis, with increased cycling of DN2 and DN3 thymocytes and expansion of the DN4 and CD8
**SP subsets.** (A) Preleukemic 6w Sur-TCR-Tg mice have abnormally small thymi, but thymic size significantly increases by 4 months of age. Absolute thymocytes counts in Sur-TCR-Tg mice and WT mice at 6 weeks and 4 months of age are shown. Each shape represents one individual mouse. Experiment was conducted three times with similar results. (B) Thymocyte subset analyses as revealed via flow cytometry in preleukemic 6w Sur-TCR-Tg mice vs. age matched WT mice. Sur-TCR-Tg mice have increased frequencies (top) and absolute numbers (bottom) of CD4−CD8− thymocytes and CD8−CD4− single positive thymocytes compared with WT mice, in contrast to diminished numbers of CD4+CD8+ double positive and CD8−CD4+ SP thymocytes. This experiment was conducted more than three times with similar results. (C) Using CD44 and CD25 to stratify the DN thymocyte subset, 6w Sur-TCR-Tg mice show a selective increase in the frequency of the DN4 subset, whereas other subsets are reduced compared to WT mice. This experiments was conducted three times with similar results. (D) BrdU incorporation demonstrates increased cycling of the DN2 (CD25+CD44+), DN3 (CD25+CD44−) and CD8+CD4−subsets in preleukemic Sur-TCR-Tg mice compared to WT controls. BrdU was injected IP to 6w Sur-TCR-Tg and control mice. Eighteen hours later, thymi were harvested and BrdU incorporation was analyzed using flow cytometry. FACS plots from one representative Sur-TCR-Tg are shown (top) as well as summative data from 7 mice. This experiment was performed twice with similar results.

**Figure 5. Signaling via the survivin reactive TCR contributes to leukemogenesis.** (A) Cell images taken from four separate cells designated as ‘high similarity scores’ vs “low similarity scores” are shown. Cells with high similarity scores were from Sur-TCR-Tg
mice and cell with low similarity scores were from WT controls. (B) Thymocytes from 6 wk Sur-TCR-Tg (black) and WT (grey) mice were harvested and stained immediately using cell surface antibodies, then fixed/permeabilized and stained with intracellular NFAT antibody. Lin− cells were gated and the frequency of DN thymocytes from Sur-TCR-Tg+ (black) and WT (grey) exhibiting nuclear translocation of NFAT is shown using the NFAT/Dapi similarity score histogram overlay. Representative result from one mouse is shown for each condition. In this analysis, a larger score indicates a greater degree of signal correlation between NFAT channel and nuclear channel and thus, translocation. Translocated cells are defined as those with a similarity score above 1. Similar results were seen in more two separate experiments containing at least 3 animals/group. (C) Thymocytes with NFAT nuclear translocation (similarity score >1.0) by ImageStream analysis in thymocyte subsets of Sur-TCR-Tg mice, compared with WT mice (5C, top). Among the DN subsets, Sur-TCR-Tg showed significantly more NFAT nuclear translocated cells in DN2 and DN4 subset compared to WT mice (5C, bottom). Similar results were obtained in two separate experiments with at least 3 animals/group. (D) Sur-TCR-Tg mice were bred with β2M−/− mice to generate Sur-TCR-Tg+β2M−/− mice, then animals were monitored for the development of T-ALL for 20 months. Kaplan-Meier analysis reveals significantly diminished death due to T-ALL in Sur-TCR-Tg+β2M−/− mice compared to those Sur-TCR-Tg+β2M+/− mice (p=0.006).

Figure 6. Sur-TCR-Tg T-ALL overexpress Notch1 and cell lines are sensitive to treatment with NOTCH inhibitors and/or cyclosporine A. (A) Immunofluorescent staining of anti-NOTCH1 antibodies in thymi of one representative pre-leukemic (left)
and leukemic (right) Sur-TCR-Tg mouse (50x magnification). Similar results were observed in > 4 animals. (B) Three separate Sur-TCR-Tg T-ALL lines were treated with a gamma secretase inhibitor (GSI), or cyclosporine A (CsA) or both agents and live cells were counted daily during a one-week culture. Stars designate cultures where significantly reduced cell counts were observed F8-3 and L8-2 using either GSI or CsA or the combination. Results are representative of four independent experiments for each cell line.
A

WT

Sur TCR-Tg Pre-leukemic

Sur TCR-Tg T-ALL

CD8
CD4
CD25
CD24
BV8
CD3

B

untreated

Sur_{20-28}

PMA/Ion

C

WT

Sur-TCR-Tg pre-leukemic

Sur-TCR-Tg T-ALL

Sur-TCR-Tg T-ALL cell line

Cui, Figure 3
A) Bar graph showing thymocytes numbers for WT, 6w, Sur-TCR-Tg 6w, WT 4m, and Sur-TCR-Tg 4m. Significance levels are indicated with p-values.

B) Flow cytometry plots comparing Sur-TCR-Tg and WT for CD4 and CD8 expression. Percentages of cells are marked for each group. The p-value for Sur-TCR-Tg pre-leukemic (n=7) compared to Wild-type (n=7) is 0.031.

C) Table showing expression levels of CD44 and CD25 in Sur-TCR-Tg+ and WT. Values are presented in a grid format.

D) Flow cytometry plots for various cell subsets (DN1, DN2, DN3, DN4, and CD8 SP) showing expression of BrdU. Significance levels for Sur-TCR-Tg pre-leukemic (n=7) and Wild-type (n=7) are indicated with p-values.
Figure 6

A.

B.

L8-3

F8-3

L8-2

Days

cell # (x10^5)

days

cell # (x10^5)

cell # (x10^5)

DMSO

5uM CsA

5uM GSI

CsA+GSI
Thymic expression of a T cell receptor targeting a tumor associated antigen co-expressed in the thymus induces T-ALL

Yongzhi Cui, Masahiro Onozawa, Haven R. Garber, Leigh Samsel, Ziyao Wang, J. Philip McCoy, Sandra Burkett, Xiaolin Wu, Peter D. Aplan and Crystal L. Mackall