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Ep-TCL1xMyc: a novel mouse model for concurrent CLL and B-cell lymphoma

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Abstract

Purpose: Aberrant Myc expression is a major factor in the pathogenesis of aggressive lymphoma, and these lymphomas, while clinically heterogeneous, often are resistant to currently available treatments and have poor survival. Myc expression can also be seen in aggressive lymphomas that are observed in the context of CLL, and we sought to develop a mouse model that could be used to study therapeutic strategies for aggressive lymphoma in the context of CLL.

Experimental Design: We crossed the Ep-TCL1 mouse model with the Ep-Myc mouse model to investigate the clinical phenotype associated with B-cell restricted expression of these oncogenes. The resulting malignancy was then extensively characterized, both from a clinical and biological perspective.

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AUTHORSHIP CONTRIBUTIONS

FL, KAR, BKH, RL and JAW designed experiments, analyzed data, wrote the manuscript and reviewed the final version. FL also planned, optimized, conducted, analyzed and interpreted all immunophenotyping and flow sorting experiments, and led sequencing experiments. BKH processed and interpreted histopathology and gross pathology. AP, RB and PY monitored quality of sequencing experiments, processed, analyzed and interpreted the data, prepared related figures and table and edited the manuscript. LY provided statistical support in the planning and analysis of experiments, and edited the manuscript. JB processed and interpreted bone marrow aspirates, prepared figures and edited the manuscript. VMG, ZAH, PK, RM, LLS, RW and DKW conducted experiments, cared for mice, and edited the manuscript. All authors reviewed the manuscript and agreed to submit it for publication.

Results: E μ -TCL1xMyc mice uniformly developed highly aggressive lymphoid disease with histologically, immunophenotypically, and molecularly distinct concurrent CLL and B-cell lymphoma, leading to a significantly reduced lifespan. Injection of cells from diseased E μ -TCL1xMyc into WT mice established a disease similar to that in the double-transgenic mice. Both E μ -TCL1xMyc mice and mice with disease after adoptive transfer failed to respond to ibrutinib. Effective and durable disease control was however observed by selective inhibition of nuclear export protein exportin-1 (XPO1) using a compound currently in clinical development for relapsed/refractory malignancies, including CLL and lymphoma.

Conclusions: The E μ -TCL1xMyc mouse is a new preclinical tool for testing experimental drug for aggressive B-cell lymphoma including in the context of CLL.

Keywords

CLL; lymphoma; mouse model; targeted therapy; ibrutinib; XPO1

Introduction

Aggressive B-cell lymphoma is a highly heterogeneous disease entity with subtypes characterized by molecular, genetic and epigenetic signatures^{1,2}. Increased Myc activity is a major oncogenic factor in several aggressive B-cell lymphomas, including Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL) and high-grade B-cell lymphomas (HGBCL)³. Aberrant Myc expression is also observed in patients with Richter's syndrome (RS)⁴⁻⁶, an aggressive B-cell lymphoma arising from chronic lymphocytic leukemia (CLL) that occurs in around 5% of CLL patients⁷. Multiple studies link increased Myc expression in lymphomas to inferior survival⁸⁻¹¹.

Novel targeted agents such as the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib and the Bcl2 inhibitor venetoclax have shown remarkable activity in CLL, but they have generally been less effective in aggressive B-cell malignancies^{12,13}. The sensitivity of DLBCL to ibrutinib is largely determined by specific genetic lesions associated with DLBCL subtypes, producing overall response rates (ORRs) of 40% in activated B-cell (ABC) subtypes, but only 5% in germinal center B-cell (GCB) DLBCL¹⁴. Alternative treatment approaches that circumvent B-cell receptor- (BCR) and apoptotic pathway-mediated resistance mechanisms are therefore of increasing clinical importance. Our group previously demonstrated efficacy of the eukaryotic nuclear export protein exportin-1 (XPO1) inhibitor selinexor in settings of ibrutinib resistance¹⁵. Other studies further support that XPO-1 inhibition has significant anti-tumor activity in pretreated or relapsed/ refractory patients with hematological malignancies¹⁶. Next-generation XPO-1 inhibitors currently in development, such as KPT-8602, exhibit similar potency but substantially lower systemic toxicities¹⁷⁻¹⁹.

Genetically engineered murine models are powerful tools to investigate the biology and therapeutic responses of lymphoid malignancies²⁰. Commonly used models of aggressive lymphoma are largely driven by overexpression of Myc²⁰. Myc under the lymphoid-specific immunoglobulin heavy chain (IgH) enhancer (E μ) gives rise to a variety of B-cell lymphomas ranging from early-onset immature Burkitt-like to late-onset DLBCLs²¹. Limitations of this model include heterogeneous phenotypes and gene expression profiles

and variable responsiveness to therapy^{22,23}. Recent studies suggest that lymphomagenesis in Eμ-Myc mice is also determined by Myc-cooperative mutations and concomitant multigenic lesions involving Cdkn2a, Nras and Kras, suggesting a two-hit pathogenesis²⁴. Combinations of Myc with other oncogenes such as Ras²⁵, oncogenic EBV components²⁶, or c-Myc-surface CD19 signaling amplification loops²⁷ have been shown to accelerate lymphoma development. However, the suitability of these models to mirror therapeutic responses to targeted treatments has not been adequately tested.

The transgenic Eμ-TCL1 mouse model is the most widely used preclinical *in vivo* platform for CLL and generally mirrors tumor-induced immune defects and therapeutic responses of aggressive unmutated human CLL²⁸. Occasional RS transformation was observed in some immune-competent Eμ-TCL1 mice as well as Eμ-TCL1 mice with conditional B-cell specific TRP53-deficiency, leading to the occurrence of highly proliferative large blastoid cells in splenic infiltrates and blood,²⁹ however, a model of simultaneous CLL and aggressive lymphoma does not exist. Given the poor survival seen in patients with RS, a model for testing therapeutics in the setting of both CLL and aggressive lymphoma would be of high interest.

We crossed Eμ-TCL1 with Eμ-Myc mice to mimic the effects of Myc overexpression in the context of CLL. Eμ-TCL1xMyc mice uniformly developed a highly aggressive lymphoid disease with features of distinct CLL and lymphoma components. While mice failed to respond to BTK inhibition with ibrutinib, durable disease control was observed with the XPO1 inhibitor KPT-8602. This provides *in vivo* proof-of-concept that Eμ-TCL1xMyc mice can serve as a therapeutic platform to test agents simultaneously against CLL and aggressive lymphoma.

METHODS AND MATERIALS

Mice and disease-related removal criteria

All animal experiments were performed under protocols approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee. Eμ-TCL1 transgenic mice developing CLL and Eμ-Myc transgenic mice developing B-cell lymphoma have been described^{21,28}. c-Myc and wild type (WT) mice were purchased from Jackson laboratories (Bar Harbor). C57BL/6J females were crossed with Eμ-Myc hemizygous males to produce Eμ-Myc hemizygous or non-transgenic (nTG) pups. Eμ-TCL1-B6 homozygous females were crossed with Eμ-Myc hemizygous males to produce Eμ-TCL1B6 hemizygous/Eμ-Myc hemizygous (abbreviated to Eμ-TCL1xMyc or double transgenic (dTG)) or Eμ-TCL1B6 hemizygous/nTG pups. To produce genetic models of inactive BTK, Eμ-Myc mice were crossed with homozygous X-linked immunodeficiency (XID)³⁰ and with Eμ-TCL1xXID mice³¹. Predefined euthanasia criteria included lethargy, difficulty walking due to spleen size, lymph node masses > 1.6cm, or loss of > 20% body weight. Survival of transgenic mice was assessed in all transgenic colony mice born within a 24 month period.

Histopathology

Organs were harvested from diseased single and double-transgenic colony mice, and diseased mice after injection of E μ -TCL1xMyc spleen cells (meeting euthanasia criteria defined above). Tissues were fixed and processed as described in supplemental methods. Staining with hematoxylin and eosin (H&E) (Leica) and Ki67 (Dako, clone TEC-3) immunohistochemistry were previously described³². For confirmatory bone marrow aspirate preparation, femurs were harvested from WT (n=3) and diseased dTg (n=3) mice. After air-drying, aspirates were fixed and stained using a modified Wright stain (Heme-3 staining set, Fisherbrand) as per manufacturer's recommendation. Photographs were taken using an Olympus SC30 camera with an Olympus BX53 microscope.

Flow cytometry

Flow optimizations and protocols are detailed in supplemental methods. Antibodies are listed in Suppl. Table 1. Murine markers of B-cell development and gating strategies followed published data and technical resource publications (Suppl. Figure 1)^{33–35}. The gating strategy and graphs from a representative WT spleen are shown in Suppl. Figure 2. All gates were set on fluorescence-minus-one (FMO) controls prepared for each individual experiment. B-cell phenotype and percentage comparisons in marrow, spleen and blood were conducted in 8 E μ -TCL1, 6 E μ -Myc and 9 E μ -TCL1xMyc (all disease requiring euthanasia), and 5 WT mice matched to the age of diseased dTg mice. B-cell light chain expression in spleen was investigated in an additional set of diseased E μ -TCL1xMyc (n=8) and non-transgenic (nTG) age-matched littermates (n=3). A third set of spleen cells from 8 diseased E μ -TCL1xMyc mice was stained with intracellular c-Myc and TCL1-A/ respective isotypes after B-cell surface staining, and corrected median fluorescence intensities (MFIs, MFI TCL1/ Myc MINUS MFI isotype PE/ AF488) were compared to those in WT spleen B cells (n=2). Only samples with >60% viability were included into statistical analyses. All data were analyzed using KALUZA software (Becton Dickinson).

Adoptive cell transfer of unselected malignant populations

Freshly harvested spleens from diseased dTG mice meeting euthanasia criteria were processed as described in supplemental methods. A dose of 1×10^7 viable splenocytes, prepared from a pool of diseased dTG donors, was injected into the tail vein of B6 WT recipient mice (n=10) at ~2 months of age. All cell suspensions contained 75% CD19⁺ B cells verified by flow cytometry. Recipients were monitored by standard hematology testing and euthanized following the criteria described above.

Flow sorting of atypical B-cell populations and adoptive transfer

CD19⁺CD5⁺ CLL-like, CD21[−]IgM[−] and CD21[−]IgM⁺ cells were sorted from a spleen single cell suspension pool of diseased dTG mice (n=3) that met euthanasia criteria. A total of 4.1×10^8 spleen cells were obtained, stained and gated as before. Flow sorting was performed on a BD FACSAriaIII sorter in purity mode, yielding a total of 1×10^6 viable CLL-like cells and 3×10^6 viable CD21[−]IgM[−] and CD21[−]IgM⁺ cells each, which were injected into tail veins of nTG littermates (n=3/population). Recipient mice were then monitored every 7-14 days using the immunophenotyping panel and gating strategy above. Mice were euthanized

once they exhibited clinical signs of overt disease, or were censored at the end of the experiment after 136 days after injection, and B-cell phenotype and light-chain expression were examined by flow cytometry.

Low-input RNA-seq of atypical B-cell populations in dTG mice

CD19⁺CD5⁺ CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ cells were flow-sorted as above from spleen suspensions from diseased dTg mice (n=3) meeting euthanasia criteria. Sorted cells were captured in PBS/10%FBS on ice and washed. Pellets were resuspended in Trizol and stored at -80°C until RNA isolation using a chloroform/ alcohol extraction method. Total RNA was quantified using the Invitrogen Qubit RNA HS Assay kit (Invitrogen). The Clontech SMARTer v4 kit (Takara Bio USA, Inc.) was used for global preamplification. Illumina sequencing libraries were derived from the resultant cDNA using the Illumina Nextera XT DNA Library Prep Kit following manufacturer's instructions. Libraries were sequenced using an Illumina HiSeq 4000 sequencer paired-end 150bp protocol to ~12 million passed filter clusters per sample. Data processing was performed according to the CLEAR workflow³⁶, which identifies reliably quantifiable transcripts in low-input RNA-seq for differentially expressed gene (DEG) transcripts using gene coverage profiles. MiXCR (v3.0.5)³⁷ was used with default parameters to identify preprocessed reads containing CDR3 regions from B-cell heavy, kappa, and lambda chains, generating a list of unique CDR3 sequences associated with their relative abundances and specific V(D)J gene usage. Global DEG analysis was performed using DESeq2 (v1.20.0)³⁸. The counts table containing CLEAR transcripts from all replicates was used for comparisons between groups. The resultant data was then used for gene-specific analysis for a targeted list of oncogenes^{1,2,39,40}. Raw FASTQ files and counts tables were deposited in GEO (Accession: GSE129515).

Drug experiments

Ibrutinib and 10% β -cyclodextrin vehicle were purchased and delivered via drinking water³¹. KPT-8602 (provided by Karyopharm, Inc.) drug (15 mg/kg/day) and vehicle (0.5% methyl-cellulose/1% tween-80) were administered by daily oral gavage¹⁸ to WT mice injected with 1×10^7 viable splenocytes, prepared from a pool of diseased dTG donors. KPT-8602/ vehicle treatment were initiated once a WBC count ≥ 8 and/or $\geq 5\%$ circulating CD19⁺CD5⁺ CLL were detected. Disease progression was monitored weekly. Mice were euthanized following criteria outlined above.

Statistical analyses

Survival was shown using Kaplan-Meier curves and curves were compared using a log-rank test. Differences between cell subset frequencies were compared using non-parametric Wilcoxon-Mann-Whitney and 1-way ANOVA (with Dunn's multiple comparison) tests assuming unequal variances or small numbers in experimental groups. Myeloblasts, promyelocytes and monocytes in bone marrow aspirates were compared using a Wilcoxon signed-rank test. R program and GraphPadPrism were used for statistical analyses.

RESULTS

E μ -TCL1xMyc mice develop an aggressive lymphoid malignancy that significantly shortens survival

Immunoblot analysis confirmed TCL1 and c-Myc expression in E μ -TCL1xMyc B cells (n=3) (Figure 1A). E μ -TCL1xMyc mice developed a malignancy at a younger age than single transgenic E μ -Myc or E μ -TCL1 mice, leading to significantly shortened survival [median survival dTG mice 46 days (range 29-74 days) *vs.* E μ -Myc 118 (42-520 days) *vs.* E μ -TCL1 368 (188-531 days), $p < 0.0001$, Figure 1B]. Respiratory distress, inguinal, axillary and intestinal lymphadenopathy were frequently observed as the malignancy progressed, and key characteristics of diseased dTG mice at endpoint were pronounced cervical lymphadenopathy and splenomegaly (Figure 1C). Further examination of tissues from WT and diseased transgenic mice characterized tumors in dTG mice as a lymphoid malignancy histologically indistinguishable from lymphoma in E μ -Myc mice, but distinct from leukemia in E μ -TCL1 mice, with infiltration of lymph nodes, liver, spleen, and marrow (Figure 1D). While pronounced spleen and liver infiltration with neoplastic lymphoid cells was seen in all transgenic models, marrow and lymph nodes were only heavily involved in Myc-driven models. Bone marrow aspirates were obtained from WT and dTG mice to confirm infiltration by large homogenous lymphoid cells in dTG compared to WT mice (Suppl. Figure 3A+B), leading to a significant reduction and impaired maturation of the myeloid and erythroid lineages (Suppl. Figure 3B–D). Taken together, dTG mice develop an aggressive lymphoid malignancy that infiltrates bone marrow and lymphoid organs and is histologically similar but clinically different to aggressive lymphoma seen in E μ -Myc mice.

Lymphoid malignancy in dTG mice exhibits immunophenotypic features of immature atypical B cells preventing physiological B-cell maturation

To characterize the model biologically, we first compared B-cell immunophenotypes and relative frequencies (percentages) from marrow, spleen, lymph node and blood in WT and diseased transgenic mice, using established murine markers of B-cell development as shown in Suppl. Figure 1^{33–35}. Due to the similarity between spleen and lymph nodes, results are only shown for spleen. The gating strategy and representative flow plots are shown in Suppl. Figure 2. We first compared broad B-cell maturation differences by evaluating IgM/IgD expression (Figure 2A) among CD5[–]B220⁺ B cells, *i.e.* after exclusion of CD5⁺ B cells with a CLL phenotype, which was expected to occur in mice carrying the TCL1 transgene. Figure 2B shows representative WT spleen IgM and IgD FMO controls. Diseased mice expressing the Myc transgene (both E μ -Myc and dTG mice) had a distinct maturation block at early B-cell development stages, altering the presence and distribution of more mature B cells in secondary lymphoid organs and blood (Figure 2C). As this arrest appeared to accumulate cells at the transitional B-cell stage (*i.e.* when B cells are developmentally intermediate between immature bone marrow and mature naïve peripheral B cells), we next compared the relative frequencies and phenotype of transitional B cells (*i.e.* CD5[–]CD93⁺B220⁺ cells), again after exclusion of CD5⁺ B cells with a CLL phenotype. E μ -TCL1xMyc mice displayed a significant enrichment in all examined organs compared to WT mice and parental strains, even in peripheral blood, where such cells are usually transient and rare (Figure 2D). However, this enriched population lacked the usual CD23 and IgM expression

patterns that typically identify physiological transitional B cells. Instead, two distinct IgM⁺ and IgM⁻ subpopulations were noticeable (Figure 2E). Overall, our results show that B-cell specific expression of TCL1 and Myc leads to the development of an immature atypical and heterogeneous B-cell population indicative of a B-cell malignancy, preventing normal physiological B-cell maturation.

Lymphoid malignancy in Eμ-TCL1xMyc mice is composed of both CLL and a heterogeneous lymphoma population with distinct IgM expression and light-chain usage patterns

To further explore the B-cell maturation block in Eμ-TCL1xMyc mice, we next investigated the distribution and frequencies of CD93⁻ cells, *i.e.* B-cells that were not described above. This revealed a significantly expanded CD19⁺CD5⁺B220⁺ CLL-like population in diseased Eμ-TCL1xMyc mice that was absent in both WT and diseased Eμ-Myc mice (Figure 3A+B **top panel**). CD19⁺CD5⁺B220⁺ CLL-like cells were however less expanded than in diseased Eμ-TCL1 mice and infiltrated predominantly blood and spleen. CD19⁺CD5⁺ cells were also detected among B220^{low} cells in all examined organs (Figure 3A+B **bottom panel**), again at lower frequencies than in diseased Eμ-TCL1 mice. Among the remaining non-CLL maturing B cells (B220⁺CD19⁺CD5⁻), the frequencies of regular follicular and marginal zone/marginal zone progenitor cells were substantially reduced in diseased Myc-expressing models, further confirming the maturation arrest described above (Figure 3C+D **left panel** for spleen, Suppl. Figure 4 for marrow and blood). Instead, atypical B cells lacking CD21 expression were substantially increased in diseased dTG mice, suggestive of an immature malignant B-cell population. These B220⁺CD21⁻ cells exhibited again both an IgM⁺ and an IgM⁻ population in dTG mice, pointing towards heterogeneity within the malignant population, whereas diseased single-transgenic Eμ-Myc mice only had a single population with IgM^{low/int} expression (Figure 3D **left panel+E**). The heterogeneity of the dTG lymphoma population was further supported by differences in IgD expression (Figure 3D **right panel**): while IgM⁻ cells also lacked IgD expression, indicative of immaturity of this population, IgM⁺ cells dimly co-expressed IgD, suggesting a more mature phenotype.

We next examined differences in light chain expression in CLL, CD21⁻IgM⁻ and CD21⁻IgM⁺ populations in 8 additional dTG mice and compared this to light chain usage in B cells from age-matched nTG littermates (n=3). B cells from nTG littermates acquired light chain expression with increasing B-cell maturation with kappa usage predominance as expected⁴¹ (Figure 3F **top row**). Light chain expression was absent in most cells in CD21⁻IgM⁻ lymphoma populations in diseased dTG mice, confirming their immature phenotype, while CD21⁻IgM⁺ cells and CD19⁺CD5⁺ CLL populations expressed predominantly kappa light chains, confirming a more mature phenotype (Figure 3F **bottom row**). All malignancy components showed higher protein expression of Myc and TCL1A than WT B cells (Suppl. Figure 5A), with highest TCL1A expression in CD19⁺CD5⁺ CLL cells, and highest Myc expression in CD21⁻IgM⁻ and CD21⁻IgM⁺ cells. Similar patterns were seen on a molecular level (Suppl. Figure 5B). Altogether, these findings suggest that dTG mice develop a malignancy with features of both CLL and lymphoma, with the lymphoma population consisting of heterogeneous subpopulation components characterized by differential IgM, light chain and oncogene expression.

RNA sequencing identifies differential V(D)J usage and confirms heterogeneity of CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ populations

We next performed RNA sequencing of flow-sorted malignant populations obtained from 3 diseased dTG mice to compare CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ B-cell populations. Post-alignment QC results are summarized in Suppl. Table 2. We first quantified the percentage of reads with heavy, kappa, and lambda chains compared to the number of total sequencing reads. The CD21⁻IgM⁻ showed the lowest and the CLL-like population the highest percentage of reads that aligned to the V(D)J region, indicating different amounts of BCRs in malignancy components (Figure 4A, Suppl. Table 3). The percentage of aligned reads was significantly different between CD21⁻IgM⁻ and CD21⁻IgM⁺ ($p=0.012$) and between CD21⁻IgM⁻ and CLL-like ($p=0.046$) cells, but not between CD21⁻IgM⁺ and CLL-like populations ($p=0.13$), suggesting a greater degree of similarity of BCR-expressing cells between CD21⁻IgM⁺ and CLL-like populations. The comparison of the top 20 most abundant CDR3 sequences and the frequency of remaining CDR3 sequences, jointly denotes as “others”, demonstrated a high degree of diversity in CD21⁻IgM⁻ cells, decreasing diversity in CD21⁻IgM⁺ cells and the lowest diversity in CLL-like populations, with light chains being more frequently used than heavy chains (Suppl. Figure 6A, Suppl. Table 4).

To examine differences in V gene usage, BCR reads were binned by specific heavy (H) and light chain V genes. Each V gene was normalized by the total number of reads corresponding to that chain. A low number and similar gene usage was seen across all populations for lambda (L) V chains (Suppl. Figure 6B, Suppl. Table 4). Kappa chain genes in contrast were highly diverse in CD21⁻IgM⁻ cells, and decreased in diversity in CD21⁻IgM⁺ and CLL-like populations, concurrent with the emergence of fewer and predominant clones (Figure 4B, Suppl. Table 4). While replicate mice largely shared the same degree of diversity for each population, there was very little overlap of distinct kappa chain V genes between different malignant populations, indicative of limited clonal relationships. Very similar patterns were observed in heavy chain genes (Suppl. Figure 6C, Suppl. Table 4). However, distinct differences were seen in transcriptome profiles of CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ populations (Figure 4C, Suppl. Table 5). A number of oncogenes implicated in CLL and lymphoma pathogenesis^{1–3,39,40} were solely upregulated in the CLL population, whereas *HES1* and *EZH2* were only overexpressed in CD21⁻IgM⁻ and CD21⁻IgM⁺ populations. Moreover, transcriptome profiles were very similar in CD21⁻IgM⁻ and CD21⁻IgM⁺ populations, with the exception of *NOTCH2*, where greater similarities existed between CD21⁻IgM⁺ and CLL. Altogether, these molecular patterns confirm differences in B-cell maturation and suggest limited clonal relationships between all malignancy components in the context of differentially expressed genetic profiles.

CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ B-cell populations establish distinct diseases identical to the engrafted malignancy in transfer experiments

We next confirmed that the E μ -TCL1xMyc malignancy was amenable to adoptive transfer by injecting pooled E μ -TCL1xMyc spleen cells from diseased donors into B6 syngeneic WT mice ($n=10$). This established a circulating malignancy 8–10 days after injection, as evidenced by increased WBC counts and/or percentages of CD19⁺CD5⁺ cells. Median survival was 38 days (range 37–42 days), and tumor histology was similar to that in dTG

mice (Figure 5A). To assess the malignant and transformation potential of different components of the dTG malignancy *in vivo*, we flow-sorted CD19⁺CD5⁺ CLL-like cells and CD21⁺IgM⁺ and CD21⁺IgM⁺ lymphoma cells from a pool of diseased dTG mice and injected them separately into nTG littermates (n=3/ population). For key characteristics of recipient mice and diseases after injection see Suppl. Table 6. All mice injected with more mature CD21⁺IgM⁺ cells died after 58-69 days with signs of fully developed lymphoid malignancy. Two of 3 mice injected with immature CD21⁺IgM⁺ cells uniformly developed lymphoid malignancy and were euthanized in poor overall condition after 58 days, with one mouse (C1457) being censored as a failed engrafter 136 days post-injection. Recipients of CLL cells survived considerably longer, potentially as a result of the lower cell dose they had received. A B220^{low/+}CD19⁺CD5⁺ CLL like population was detected in blood of all CLL-cell recipients, with varying spleen involvement and the least infiltration in marrow (Figure 5B, Suppl. Figure 7). The phenotype of remaining and relatively reduced B220⁺CD5⁺ maturing B cells was CD21^{int}IgM^{-/int}, consistent with a population of non-malignant follicular B cells, and there was no evidence of B220⁺ cells with CD21/IgM expression patterns seen in dTG mice (Suppl. Figure 8). Mice with disease after injection of immature CD21⁺IgM⁺ lymphoma cells developed a B220⁺CD5⁺CD19^{int}CD21^{int}IgM^{-/int} population, especially in spleen, representing follicular B cells, and a B220⁺CD5^{int}CD19⁺CD21⁺IgM⁺ population identical to the phenotype of engrafted lymphoma cells (Figure 5C, **left panel**). CD21⁺IgM⁺ cells and a frank CD19⁺CD5⁺ CLL-like population were not detected (Suppl. Figure 7+8). All recipients of CD21⁺IgM⁺ cells lacked CD19⁺CD5⁺ CLL-like cells, but developed a B220⁺CD5^{-/int}CD19^{int/+} population (Figure 5C, **right panel**, Suppl. Figure 7+8). This could be further divided into CD21^{int}IgM^{-/int} follicular B cells, CD21⁺IgM⁺ lymphoma cells, and a considerably larger population of CD21⁺IgM⁺ lymphoma cells. The CLL-like population in CLL recipients and non-malignant follicular B cells in all donor groups preferentially expressed kappa light chains (Figure 5D). Kappa also remained the predominantly expressed light chain in all lymphoma populations in CD21⁺IgM⁺ recipients. In contrast, CD21⁺IgM⁺ lymphoma cells in CD21⁺IgM⁺ recipients largely lacked light chain expression. Altogether, although based on small numbers, these results demonstrate that recipients of a distinct component of the dTG malignancy develop a malignancy reflecting the characteristics of injected disease, providing *in vivo* support of the molecular patterns described above.

dTG mice are resistant to BTK inhibition but respond to XPO-1 inhibition

To characterize the dependence of a Myc-driven malignancy on BTK-mediated B-cell receptor signaling *in vivo*, Eμ-Myc mice were crossed with XID and Eμ-TCL1xXID mice. In contrast to the Eμ-TCL1 model, where crossing of TCL1 with XID mice with inactive BTK prolongs survival and reduces CLL burden³¹, genetic BTK inactivity did not improve survival in Myc-expressing mice, suggesting Myc involvement in mediation of resistance to BTK inhibition [median survival Eμ-Myc mice (n=109) 118d (range 42-520) *vs.* Eμ-MycxXID mice (n=37) 78d (50-123), *p*=1.0; Eμ-TCL1xMyc (n=68) 46d (29-74) *vs.* Eμ-TCL1xXIDxMyc (n=38) 52d (36-73), *p*=0.172; Figure 6A]. Intrinsic resistance to BTK inhibition was then confirmed pharmacologically. Compared to vehicle, treatment with ibrutinib from the date of weaning did not improve survival in either myc-carrying transgenic strains (*p*=0.83 and *p*=0.88, respectively, Figure 6B+C). XPO-1 inhibitors have

successfully been used in ibrutinib-refractory mice and cell lines^{15,18}, partly by inhibition of activation of downstream B-cell receptor targets and suppression of BTK gene expression³². As a proof of concept we therefore treated WT mice with evidence of circulating disease after injection of spleen cells from a pool of fully diseased dTG donors with XPO-1 inhibitor KPT-8602 (n=7) or vehicle (n=10). Median survival in vehicle-treated mice was 25 days (range 17-29 days), whereas all KPT-8602 treated mice survived until day 75 when the study was terminated ($p<0.0001$, Figure 6D). After initiation of KPT-8602, WBC counts decreased throughout treatment, while continuing to rise in mice receiving vehicle (Figure 6E **left panel**), mostly driven by increasing percentages of circulating CD19⁺CD5⁺ CLL cells (Figure 6E **right panel**). Spleen weights were significantly lower ($p=0.0025$) in KPT-8602 than in vehicle treated mice (Figure 6F). Lymph nodes and spleens were effaced with diffusely proliferating Ki67⁺ neoplastic lymphocytes in vehicle treated mice (Figure 6G). This experiment not only confirms the suitability of the adoptive transplant Eμ-TCL1xMyc mouse model for therapeutic exploration, but provides additional support for the evaluation of XPO-1 inhibitors in aggressive lymphomas.

DISCUSSION

We here report a new double transgenic Eμ-TCL1xMyc-driven model of aggressive B-cell lymphoma that offers several advantages over existing lymphoma models. These include high penetrance of very aggressive disease, reproducible and consistent histology and immunophenotype, amenability to adoptive transfer, uniformly shortened survival duration, and possibility of studying this lymphoma in the context of co-existent CLL. Clinical responses to ibrutinib in DLBCL are largely determined by subtype and the presence of additional BCR mutations¹⁴. Importantly, a recent phase 3 study reported that the addition of ibrutinib to standard immunochemotherapy did not improve event-free survival and increased toxicity in DLBCL patients, suggesting that BCR pathway inhibition may not be a viable treatment strategy in this setting⁴².

Extensive profiling studies have demonstrated that molecular alterations as well as genetic and epigenetic signatures can be used to classify different lymphoma subtypes and to predict responses to treatment¹. Similar strategies have been employed to characterize the patterns of genetic evolution leading from CLL to lymphoma transformation, and to characterize the genetic landscape in patients with CLL and clonally related lymphomas compared to CLL and *de novo* DLBCL^{5,6}. The Eμ-TCL1xMyc mouse model reported here is based on the malignant potential of just two known oncogenes, TCL1 and Myc, which might be an oversimplification of the complex and dynamic landscape of genetic and epigenetic alterations. However, Myc dysregulation occurs in several subtypes of aggressive B-cell lymphomas, and has also been described in independent cohorts of RS patients⁴⁻⁶, highlighting Myc as a major pathogenic factor. Lymphomas characterized by Myc in combination with *BCL2* and/ or *BCL6* rearrangements are currently classified as HGBCL³, and recent studies highlight the poor outcome of patients with HGBCL^{8,9}. To further characterize the molecular signatures in the three malignancy components developing in Eμ-TCL1xMyc mice, we performed RNA sequencing and employed a candidate-gene approach to compare differential expression of oncogenes with implications in DLBCL, CLL and RS^{1,2,39,40}. Typical CLL-related oncogenes such as *IRF4*, *Rb1*, *RelA* and *CD79b* were

solely upregulated in the CLL population, whereas *HES1*, a regulator of Notch-signaling, and *EZH2*, a molecular marker of the EZB genetic subtype², were only overexpressed in CD21⁻IgM⁻ and CD21⁻IgM⁺ lymphoma populations. While candidate genes were similarly expressed in the two lymphoma populations, we observed some overlap between the CLL and the IgM⁺ population for *NOTCH2* expression. *NOTCH2* was recently found to be a marker of the BN2 DLBCL subtype², and also controls non-autonomous Wnt-signaling in CLL⁴³. Together, these molecular fingerprints confirm distinct properties of different malignant populations developing in Eμ-TCL1xMyc mice, although further studies will be needed for a comprehensive molecular characterization.

Moreover, the mechanisms by which TCL1 and Myc co-expression accelerate disease and lead to a phenotype of concurrent CLL and heterogeneous lymphoma are unknown and should be further explored. We observed higher TCL1 expression in the CLL and higher Myc expression in the lymphoma populations at both the protein and transcriptional levels, suggesting that the degree of disease expansion or the predominance of a disease component is primarily driven by one of the two oncogenes. However, TCL1 is a known activator of the PI3k-Akt (PKβ) oncogenic pathway⁴⁴ and also interacts with a variety of regulators of cell survival such as AP1 and Atm affecting NFκB and IKBα⁴⁵. Interactions between TCL1 and Myc and the mechanisms leading to changes in proliferation, cell cycle, and apoptosis are currently not understood, and might be different in the distinct malignancy components in our studies.

An important question is whether Eμ-TCL1xMyc mice can be proposed as a model of a specific aggressive B-cell lymphoma. To determine clonality patterns, and as a method to assess whether this model is of a lymphoma transformed from a CLL clone or two distinct co-occurring malignancies, we conducted V(D)J sequencing and adoptive transfer experiments of flow-sorted malignancy components. V(D)J sequencing confirmed substantial differences in the B-cell maturation stage, *i.e.* the amount of cells expressing a complete BCR, and the diversity of V gene usage, with CD21⁻IgM⁻ cells being the least mature but most diverse and CLL-like cells being the most mature but least diverse population. Clear clonality patterns were absent, demonstrating that this is not a model of clonally-related RS. Taken together, though, our molecular data indicate that Eμ-TCL1xMyc mice are a compound model of aggressive lymphoma and CLL, mimicking at least one therapeutic response of patients with lymphoma subtypes unresponsive to BCR-targeted agents to XPO-1 inhibition.

XPO-1 inhibitors have demonstrated significant pre-clinical activity in various hematological malignancies, even in the setting of ibrutinib resistance^{15–19}. As a result, several phase Ib/II clinical studies are currently being conducted in various malignancies, including CLL and aggressive lymphoma^{46–48}. Another clinical study is currently under way to examine the combination of selinexor with ibrutinib in patients with relapsed/refractory CLL or aggressive lymphoma⁴⁹. Eμ-TCL1xMyc mice with circulating disease after adoptive transfer of Eμ-TCL1xMyc cells showed rapid and lasting responses to treatment with the XPO-1 inhibitor KPT-8602. These findings provide further evidence that XPO-1 inhibition is a valid therapeutic strategy to pursue for patients with resistance to BCR-pathway inhibitors, and proof-of-principle that the Eμ-TCL1xMyc model is a potential tool to

optimize treatment strategies aimed at XPO-inhibition, especially in combination with other agents. Taken together with data derived from patient cohorts and ongoing clinical studies, future studies using the murine model described here have the potential to identify pathways of diagnostic and therapeutic relevance. This is urgently needed as novel agents targeting the B-cell receptor pathway are mostly unsuccessful in patients with aggressive lymphoma. Altogether, we expect that the Eμ-TCL1xMyc mouse model will prove a useful and predictable resource to further study the disease biology of aggressive lymphomas as well as the activity of novel therapeutic strategies in this setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DISCLOSURE OF CONFLICTS OF INTEREST

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Statement of translational relevance

Patients with Myc-driven aggressive B-cell lymphoma and those with concomitant aggressive lymphomas and CLL have poor outcomes and are often unresponsive to currently available therapeutics. Uniform and predictable Myc-driven mouse models are lacking, and existing mouse models mostly fail to mimic therapeutic responses to targeted agents observed in patients. Using B-cell specific overexpression of two known oncogenes of CLL and aggressive lymphoma, we created E μ -TCL1xMyc mice that uniformly develop aggressive B-cell lymphoma with concurrent CLL. E μ -TCL1xMyc mice have intrinsic resistance to BTK inhibition, but respond to a nuclear export (XPO-1) inhibitor currently in clinical development for relapsed/refractory malignancies, including CLL and aggressive lymphoma. Our studies demonstrate that E μ -TCL1xMyc mice can be a tool to evaluate the biology and treatment of Myc-driven aggressive lymphomas in the setting of CLL.

Figure 1: Comparison of survival and disease characteristics between E μ -Myc, E μ -TCL1 and E μ -TCL1xMyc mice:

(A) Immunoblot analysis from negatively isolated CD19⁺ cells from spleens taken from disease-free WT B6 (n=2) and diseased E μ -TCL1, E μ -Myc and E μ -TCL1xMyc mice (n=3 each) meeting euthanasia criteria (B) Survival of mice was assessed in all transgenic colony mice born within a 24-month period and compared using a log-rank test. (C) Gross assessments and necropsies were conducted in all mice throughout the course of all experiments. Representative photographs (taken with Nikon D5100 camera) showing lymph

nodes (LN) and spleens from WT and E μ -TCL1, E μ -Myc and E μ -TCL1xMyc mice with disease meeting euthanasia criteria. **(D)** Histopathological assessments were conducted in WT (n=2) and E μ -TCL1 (n=3), E μ -Myc (n=6) and E μ -TCL1xMyc (n=5) colony mice with disease meeting euthanasia criteria. All photographs were taken using an Olympus SC30 camera with an Olympus BX53 microscope. Normal LN architecture is replaced by sheets of large lymphocytes, interspersed mitotic figures (M above each mitosis), and tingible body macrophages (T above each macrophage) in E μ -Myc and E μ -TCL1xMyc mice. Normal spleen architecture is effaced by neoplastic cells in all transgenic mice. Expansion of splenic white pulp by neoplastic lymphocytes can be seen as white foci in enlarged spleens, whereas white (W) and red pulp (R) areas are clearly distinguished in WT mice. In livers of all transgenic mouse strains neoplastic lymphoid cells (arrowhead) expand portal triads (P) and are seen circulating within sinusoids (arrows). Bone marrow infiltration with lymphoid cells is severe in E μ -Myc and E μ -TCL1xMyc mice, while E μ -TCL1 mice contain many areas of normal hematopoietic marrow (N).

Figure 2: Immunophenotypic comparison of broad B-cell maturation and immature B-cell stages:

B-cell immunophenotype was examined in marrow, spleen and blood from WT (n=5) and diseased Eμ-TCL1 (n=8), Eμ-Myc (n=6), and Eμ-TCL1xMyc (n=9) mice (all transgenic mice with disease requiring euthanasia) using established murine markers of B-cell development and gating strategies^{33–35}. Cells were gated on viable, single, CD3⁻CD11b⁻ non-T/ non-myeloid cells, and further dissected according to B220, CD93, CD5, CD19, CD21, CD23, IgM and IgD expression. (A) General B-cell maturation stages were defined using dynamic IgM and IgD expression after exclusion of CD5⁺ B cells with a CLL

phenotype to avoid “contamination” with CLL-like cells. **(B)** Representative flow plots of FMO controls for IgM and IgD from a WT spleen sample. **(C)** Representative flow plots showing IgM and IgD expression and maturation differences in marrow, spleen and blood in WT and transgenic mice. *Note:* flow cytometry was focused on E μ -Myc mice with early disease manifestation. **(D)** Relative frequencies of CD5⁻CD93⁺B220⁺ B cells, gated on CD11⁻CD3⁻ non-myeloid and non-T cells, were compared in marrow, blood and spleen between WT and diseased transgenic mice requiring euthanasia using 1-way ANOVA with Dunn’s multiple comparison and non-parametric tests. All graphs show mean $\pm$ standard error of mean (SEM). **(E)** Representative flow plots from spleen demonstrating atypical CD23 and IgM expression patterns on CD5⁻CD93⁺B220⁺ cells in Myc bearing mice, characterized by loss of CD23 and development of distinct IgM⁺ and IgM⁻ subpopulations.

Figure 3: Immunophenotypic comparison of maturing B-cell stages:

B-cell immunophenotype was further examined in marrow, spleen and blood from WT and diseased Eμ-TCL1 (n=8), Eμ-Myc (n=6), and Eμ-TCL1xMyc (n=9) mice (all transgenic mice with disease requiring euthanasia) using established murine markers of B-cell development and gating strategies^{33–35}. Relative frequencies were compared using 1-way ANOVA with Dunn's multiple comparison and non-parametric tests. **(A)** Relative frequencies of CD19⁺CD5⁺ CLL-like cells gated on B220⁺ and B220^{low} cells in marrow, blood and spleen. **(B)** Representative flow plots from spleens showing CD19⁺CD5⁺ CLL-

like cells gated on maturing B220⁺ and B220^{low} cells. **(C)** Relative frequencies of follicular B cells (Foll BC) and marginal zone/ marginal zone progenitor cells (MZ/MZP) among B220⁺CD5⁻ maturing B cells in spleen (see Suppl. Figure 4 for marrow and blood). **(D)** Representative flow plots from spleen demonstrating CD21 vs. IgM (left column), and IgD vs. CD23 expression (right column; for characterization of FOLL and MZ/MZP cells in disease-free mice). Plots in the right column are overlaid dot plots to demonstrate the phenotype of FOLL (red) and MZ/MZP cells (blue) in WT mice and their atypical expression in diseased mice. Myc-expressing mice acquire a predominantly CD21^{low/-} phenotype, with dTg mice developing a CD21⁻IgM⁺IgD^{interm} (green, right column) in addition to a CD21⁻IgM⁻IgD^{-dim} population (black, right column), whereas Eμ-Myc mice predominantly have a single population with CD21⁻IgM^{low/intermediate} expression. **(E)** Relative frequencies of CD21⁻ maturing B cells with IgM⁻ and IgM⁺ expression in diseased Eμ-Myc and dTg mice. **(F)** Kappa and lambda light chain expression in pre-pro, transitional (trans) and follicular (FOLL) spleen B cells in non-transgenic (nTG) littermates (n=3) and CD21⁻IgM⁻, CD21⁻IgM⁺ and CD19⁺CD5⁺ CLL populations in diseased dTg (n=8) mice, with representative flow plots.

Figure 4: BCR reads and transcriptome profiles derived from low-input RNA-seq:

Low-input RNA sequencing was performed on flow-sorted CLL-like, CD21⁻ IgM⁻ and CD21⁻ IgM⁺ B-cell populations from 3 diseased dTG mice, and data processing was performed according to the CLEAR workflow³⁶. (A) Mean percentage of sequencing reads aligned to BCR V(D)J gene segments using MiXCR³⁷ in CD21⁻ IgM⁻, CD21⁻ IgM⁺ and CD19⁺ CD5⁺ CLL-like samples with SEM error bars. Group comparisons were calculated using a Welch's t-test. * - $p < 0.05$; ns – not significant. BCR read information is also shown in Suppl. Table 3. (B) BCR reads were binned by kappa light chain V gene names. Each V

gene was normalized by the total number of kappa-chain reads. The top 10 most abundant genes are shown by replicate with shared V genes having the same color across all replicates and samples. Genes not in the top 10 are grouped as “others” (bottom-most segment in each stacked-bar). Gene names of the top 10 V genes in order of abundance are presented in Suppl. Table 4. (C) Heatmap showing the DESeq2 normalized expression for selected candidate oncogenes plus TCL1 and Myc. Genes of interest were assessed by CLEAR for robustness. Only differentially expressed CLEAR transcripts (Benjamini-Hochberg corrected DESeq2 p-value <0.05) were used in heatmap generation. For ease of visualization, the color scale of each column was adjusted by column minimum and maximum. DESeq2 normalized expression, log2-fold change, and p-values and adjusted p-values are presented in Suppl. Table 5.

Figure 5: Adoptive transfer of Eμ-TCL1xMyc spleen cells and separate CLL-like, CD21⁻IgM⁻ and CD21⁻IgM⁺ B-cell populations into nTG recipients:

(A) Gross pathology and histology of lymph nodes and spleens from WT mice injected with whole spleen cells from a pool of diseased Eμ-TCL1xMyc mice meeting euthanasia criteria (“engrafted”, n=10) and Eμ-TCL1xMyc colony mice. Histopathological assessments were conducted in 6 injected WT mice with disease meeting euthanasia criteria and colony mice as described in Figure 1D. (B) B220^{low/+}CD19⁺CD5⁺ CLL-like population (gated on viable single CD3⁺CD11b⁻CD93⁻ mononuclear cells [MNCs]) and relative reduction of non-

malignant B220⁺ B cells (gated on viable single CD3⁻CD11b⁻CD93⁻CD19⁺CD5⁻ MNCs) in recipients of CD19⁺CD5⁺ cells (n=3, donor cells obtained by flow sorting a pool of spleens from 3 fully diseased Eμ-TCL1xMyc mice). **(C left panel)** CD21⁻IgM⁻ expansion (gated on viable single CD3⁻CD11b⁻CD93⁻B220⁺CD19⁺CD5⁻ MNCs) in 2 of 3 recipients of CD21⁻IgM⁻ cells. **(C right panel)** CD21⁻IgM⁺ expansion and moderate CD21⁻IgM⁻ expansion (gated on viable single CD3⁻CD11b⁻CD93⁻B220⁺CD19⁺CD5⁻ MNCs) in recipients (n=3) of CD21⁻IgM⁺ donor cells. **(D)** Comparison of light chain usage in malignancies in all recipients (n=9): preferential kappa light chain usage in CLL-like population in CLL recipients and in lymphoma populations in CD21⁻IgM⁺ recipients, and lack of light chain expression in CD21⁻IgM⁻ lymphoma populations in CD21⁻IgM⁻ recipients.

Figure 6: dTG mice show intrinsic resistance to BTK inhibition but response to alternative pathway targeting via nuclear export inhibition:

(A) Survival was assessed in E μ -Myc and E μ -TCL1xMyc mice crossed with XID mice, rendering BTK inactive in all BTK expressing cells, and compared to transgenic colony mice born within a 24 month period (see Figure 1B) using a log-rank test. (B and C) E μ -Myc and E μ -TCL1xMyc mice were treated with ibrutinib (n=10/strain) or vehicle (n=10/strain) from date of weaning³¹ and survival was compared using a log-rank test. (D) Treatment of WT mice with disease after injection of 1×10^7 spleen cells from a pool of

diseased Eμ-TCL1xMyc donors with 15mg/kg/day nuclear export inhibitor KPT-8602 (n=7) or vehicle (n=10) was initiated upon WBC count ≥ 8 and/or $\geq 5\%$ peripheral CD19⁺CD5⁺ cells, and survival was compared with a log-rank test. (E) Comparison of WBC counts (number of WBCs/ 100 cells/ high-powerfield [hpf], 60Xobjective, averaged across 4-6 hpfs) and percentage of CD19⁺CD5⁺ CLL cells in blood (assessed by flow cytometry) after initiation of treatment with KPT-8602 or vehicle by oral gavage using a non-parametric Wilcoxon-Mann-Whitney test. (F) Comparison of spleen weights between all vehicle-treated and 5 KPT-8602 treated mice where spleen weights were measured at endpoint using a non-parametric Wilcoxon-Mann-Whitney test. (G) Histopathological assessments were conducted in 6 vehicle-treated and 4 KPT-8602-treated mice. Lymph nodes from vehicle-treated mice were effaced with neoplastic lymphocytes. In KPT-8602-treated mice, lymph nodes were devoid of neoplasia and retained clear cortical (C) and medullary architecture (M). Diffuse immunoreactivity for Ki67 in neoplastic cells was found in vehicle treated but not KPT-8602 treated mice. Normal spleen architecture was distorted by infiltration with sheets of lymphoma cells in vehicle treated mice, while KPT-8602-treated mice had few neoplastic cells with clear white pulp (W) and red pulp (R) areas. Neoplastic cells in spleens of vehicle mice were diffusely Ki67 positive. In KPT-8602 treated mice, positive cells were limited to the red pulp and corresponded to areas of extramedullary hematopoiesis. Photographs of histology were taken using an Olympus SC30 camera with an Olympus BX53 microscope.