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ORIGINAL ARTICLE

Re-activation of mitochondrial apoptosis inhibits T-cell lymphoma survival and treatment resistance

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T lymphocyte non-Hodgkin's lymphoma (T-NHL) represents an aggressive and largely therapy-resistant subtype of lymphoid malignancies. As deregulated apoptosis is a frequent hallmark of lymphomagenesis, we analyzed gene expression profiles and protein levels of primary human T-NHL samples for various apoptotic regulators. We identified the apoptotic regulator MCL-1 as the only pro-survival BCL-2 family member to be highly expressed throughout all human T-NHL subtypes. Functional validation of pro-survival protein members of the BCL-2 family in two independent T-NHL mouse models identified that the partial loss of *Mcl-1* significantly delayed T-NHL development *in vivo*. Moreover, the inducible reduction of MCL-1 protein levels in lymphoma-burdened mice severely impaired the continued survival of T-NHL cells, increased their susceptibility to chemotherapeutics and delayed lymphoma progression. Lymphoma viability remained unaffected by the genetic deletion or pharmacological inhibition of all alternative BCL-2 family members. Consistent with a therapeutic window for MCL-1 treatment within the context of the whole organism, we observed an only minimal toxicity after systemic heterozygous loss of *Mcl-1 in vivo*. We conclude that re-activation of mitochondrial apoptosis by blockade of MCL-1 represents a promising therapeutic strategy to treat T-cell lymphoma.

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INTRODUCTION

T lymphocyte non-Hodgkin lymphoma (T-NHL) accounts for approximately 10% of all NHL.[1] It comprises a group of diverse lymphoma entities of T lymphocyte or less frequently natural killer/T-cell origin.[1–13] Characterization of different T-NHL subtypes is notoriously difficult and recurrent genetic aberrations are rare.[1,14–16] Gene expression profiling has substantially advanced our understanding of the molecular complexity and has added a novel diagnostic tool to differentiate between morphologically similar T-NHLs.[2–13,17–22] Despite substantial progress in the molecular understanding of different T-NHL subgroups, the mechanisms of disease initiation and maintenance are still not well understood.[21,23]

The interaction between pro-apoptotic and antiapoptotic members of the BCL-2 protein family largely controls apoptosis,[23,24] which is efficiently blocked in T-NHL cells.[21,23,25] Pro-apoptotic BH3-only proteins such as BIM, PUMA and BID can directly or indirectly activate the pore-forming pro-apoptotic BCL-2 family members BAX or BAK at the outer mitochondrial membrane to facilitate caspase activation. This process is inhibited by the antiapoptotic BCL-2 family members, such as BCL-2, BCL-X_L or MCL-1.[24,26] Pro-survival BCL-2 proteins potently protect lymphoma cells of B-cell origin;[24,27–32] however, their individual role during T-cell lymphomagenesis in humans remains largely undefined.

T cells express MCL-1 throughout development and require MCL-1 for the survival of T-cell progenitors and mature T cells.[33–36] BCL-2 is expressed only in early $CD4^-/CD8^-$ thymocytes and in $CD4^+$ or $CD8^+$ peripheral T cells;[37,38] however, its expression is essential only for mature T cells.[30,39,40] BCL-X_L protects $CD4^-/CD8^-$ thymocytes,[41–43] but it is dispensable for activated T cells despite its elevated expression.[35,43]

The critical role of BCL-2 proteins for T lymphocytes suggests a similar contribution also for transformed T cells. Using an irradiation-induced T-cell lymphoma mouse model,[26,34,35] deletion of the pro-apoptotic BH3-only proteins BIM and BAD were reported to accelerate T-NHL development.[27] In the context of p53 deficiency, MCL-1 was recently shown to contribute to the survival of thymic lymphoma cells in mice.[1,44]

Little evidence exists about the functional relevance of individual antiapoptotic BCL-2 family members across the board of human T-cell lymphoma subtypes. Here we identified a consistently high expression of MCL-1 in human T-NHL subsets in primary patient samples both on mRNA and protein level. The functional validation showed that the development and maintenance of T-NHL critically required MCL-1 for lymphoma cell survival *in vivo*. Moreover, we show that a therapeutic window for the inhibition of MCL-1 exists within the context of the whole organism.

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MATERIALS AND METHODS

In silico integrative analysis of gene expression data set on human T-NHL

Data from 15 array-based gene expression profiling studies[2–13,18–21] (see Supplementary Figure S1a) with primary transcriptome data sets on different human T-NHL subsets were integrated and summarized on a heat map focusing on apoptotic regulators. The gene expression profilings were all conducted by hybridization on either *Affymetrix Human Genome U133 Plus 2.0 Arrays [HG-U133_Plus_2]* or *[HG-U133A] Affymetrix Human Genome U133A Array*. All Affymetrix-platform-derived data sets were separately background-corrected and preannotated using the BioConductor package 'affy' in R 3.0.0. Replicates were combined with their original samples and the mean of both was calculated. After that, the data set was quantile-normalized. Genes were annotated (updated) via Ensembl ID using 'biomaRt'. Probe sets of a gene that map to more retained/dysfunctional transcripts than other probe sets of the same gene were removed from our analysis. The residual ambiguous probe sets assigned to a gene were then reduced by calculating their mean.

Positron emission tomography (PET)

PET analysis was performed on recipient mice on days 16 and 18 after T-NHL cell transplantation. ^{18}F-fluorodesoxyglucose (^{18}F-FDG) was synthesized as previously described[45] and was obtained from the radiopharmacy unit of the TU Munich. Imaging was carried out using a dedicated micro-PET/CT system (Inveon, SIEMENS Preclinical Solutions, Munich, Germany). Before administration of the tracer, the mice were anesthetized with Isoflurane. Subsequently, 100 µl ^{18}F-FDG with an activity dose of 5–10 MBq was administered via tail vein injection. The accumulation of the radiotracer in the lymphoma-burdened tissues was allowed for 45 min. Subsequently, mice were imaged for 15 min (static data acquisition).

RESULTS

MCL-1 is the sole antiapoptotic BCL-2 family member to be highly expressed across various human T-NHL subtypes

We analyzed array-based mRNA expression profiles from 15 technically comparable, publicly available *in silico* data sets on various cohorts of primary human T-cell lymphoma subtypes (including T lymphoblastic leukemia; simplified as 'T-NHL') together constituting the largest T-NHL data set available[2–13,18–20] (Supplementary Figure S1a). The summarized data were illustrated in a heat map based on differential mRNA expression as indicated by a color-coded histogram that shows high expression as red color and low expression as white color compared with the transcriptome median (Supplementary Figure S1b). We focused on pro-apoptotic and antiapoptotic members of the BCL-2 family to understand which one of them might constitute a promising target for T-NHL therapy.

We identified *MCL-1* as the sole pro-survival BCL-2 family member showing a high expression throughout healthy T-cell subsets as well as all human T-NHL subtypes ranging from immature acute lymphoblastic leukemia to mature peripheral T lymphocyte lymphoma (PTCL). The lack of relevant expression of BCL-2 or BCL-X_L throughout all T-NHL subtypes pointed toward a functional relevance of MCL-1 for T-NHL cell survival. MCL-1 was highly expressed even in T-NHL subtypes that originate from less frequent subsets, such as natural killer/T-cell lymphoma, hepatosplenic T-NHL or angioimmunoblastic T-cell lymphoma (AITL)[14,15] (Figure 1a). Elevated expression of *MCL-1* was restricted to the antiapoptotic splice variant, namely MCL-1long, but not to the short pro-apoptotic form of MCL-1 (MCL-1short) (Figure 1a). Despite the heterogeneity of T-NHL represented in the gene expression data, we found that none of the other antiapoptotic BCL-2 family members with the exception of *BCL2A1* (A1) was expressed at relevant levels when compared with *MCL-1*.

Despite the identification of MCL-1 as the most prominently expressed pro-survival BCL-2 family member in human T-cell lymphoma, it is noteworthy that its expression levels did not differ substantially between healthy and malignant T-cell subsets. This suggested that the contribution of MCL-1 to T-cell survival remained important even after T cells underwent malignant transformation. To substantiate the correlation between mRNA and protein expression levels in human samples especially in the light of the short half-life and high turnover rate of MCL-1,[17] we tested the protein expression of MCL-1 in comparison to BCL-2 in histological specimen from a cohort of lymph node biopsies of patients with the most common subtypes of nodal T-NHL, such as AITL, anaplastic large cell lymphoma (ALCL) or PTCL, not otherwise specified (PTCL-NOS). These analyses revealed high-level expression of MCL-1 protein in 66.6% (14/21) of PTCL-NOS specimens, in 100% (8/8) of ALCL sections and in 100% (7/7) of AITL samples (Figures 1b and c, Supplementary Figure S1c). In contrast, in most cases of PTCL-NOS or AITL, BCL-2 expression was virtually absent and only half of the ALCL samples showed strong BCL-2 expression.

This analysis performed on primary human T-NHL samples supported the notion that MCL-1 is a critical antiapoptotic protein in T-NHL.

Loss of a single allele of Mcl-1 delays tumor development in an ITK-SYK-driven T-cell lymphoma mouse model

We tested whether MCL-1 protected lymphoma cells during the process of malignant transformation. We took advantage of a mouse model based on the inducible expression of the patient-derived fusion kinase ITK-SYK.[23] In this model, CD4-Cre-inducible expression of *ITK-SYK* from the Rosa26 locus drives the development of a green fluorescent protein-positive (GFP$^+$) mature T-NHL in *Rosa26-lox-STOP-lox ITK-SYK$^{ki/+}$ CD4Cre$^{+/-}$* mice.[23] We crossed those mice with mice harboring floxed alleles of *Mcl-1* to obtain *Mcl-1$^{fl/+}$Rosa26-lox-STOP-lox-ITK-SYK$^{ki/+}$ CD4Cre$^{+/-}$* (referred to as *Mcl-1$^{fl/+}$IS$^{+/-}$CD4Cre*) mice and respective controls. Consistent with previous reports,[23] we observed diminished numbers of TCRβ$^+$ cells 4 weeks after birth in *IS$^{+/-}$CD4Cre* mice, which is caused by the constitutive ITK-SYK-dependent signaling and subsequent negative selection of double-positive thymocytes during thymic development (Figure 2a). This phenotype was aggravated in both *Mcl-1$^{fl/+}$IS$^{+/-}$CD4Cre* and *Mcl-1$^{fl/fl}$IS$^{+/-}$CD4Cre* mice causing a further reduction in peripheral T-cell numbers when one or both alleles of *Mcl-1* were deleted. This suggested that MCL-1 protects thymocytes from apoptosis during the process of thymic selection as well as protecting mature T cells in the periphery (Figure 2a).

At 16 weeks after birth, the *IS$^{+/-}$CD4Cre* mice showed a prominent increase in TCRβ$^+$ cells in the peripheral blood (PB) consistent with the development of aberrant T-cell populations and of GFP$^+$ lymphoma.[23] In contrast, experimental mice deficient for one allele of *Mcl-1* (*Mcl-1$^{fl/+}$IS$^{+/-}$CD4Cre* mice) failed to show a comparable elevation in PB T-cell numbers (Figure 2b). *Mcl-1*-targeted mice presented with TCRβ$^+$ cells in the PB comparable to those seen in control mice, effectively counterbalancing the oncogenic signaling emanating from ITK-SYK (Figure 2b). This reduction in aberrant T-cell numbers in the PB was consistent with a significantly delayed lymphoma onset and prolonged survival in *Mcl-1$^{fl/+}$IS$^{+/-}$CD4Cre* mice (Figure 2c; $P = 0.001$). Lymphoma-burdened mice showed a strong infiltration of GFP$^+$ T cells in the spleen, lymph nodes, bone marrow and PB consistent with T-NHL development (Supplementary Figure S2). In line with a tightly regulated and efficient deletion of the floxed allele of *Mcl-1*, we observed the recombination of the loxP-targeted *Mcl-1* allele and a corresponding reduction in MCL-1 protein levels in lymphoma cells from *Mcl-1$^{fl/+}$IS$^{+/-}$CD4Cre* mice compared with control cells (Supplementary Figures S2b and e).

The heterozygous deletion of *Mcl-1* significantly prolonged lymphoma-free survival of mice compared with *Mcl-1*-proficient mice, which supported the notion that MCL-1 is a critical pro-survival protein for malignant T cells. Of note, the protection was

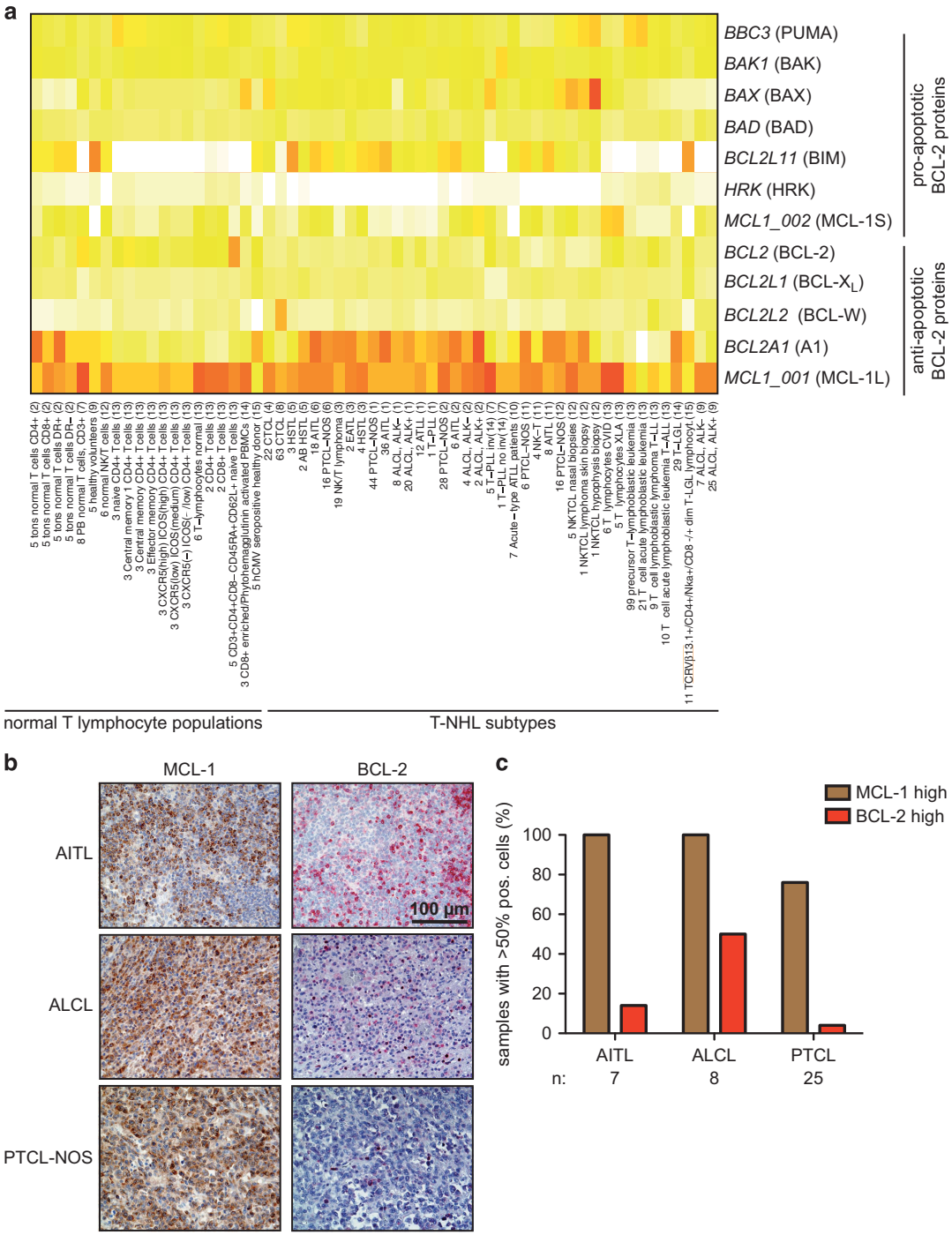

Figure 1. High expression of MCL-1 in human T-cell lymphoma subtypes. (**a**) Array-based gene expression data retrieved from publically available primary *in silico* data sets of whole-genome profilings. High-expression intensity is depicted in red and low expression in white. High expression is defined as above and low expression as below the transcriptome median, respectively. On the *x* axis, the first digit(s) indicate(s) the number of samples per entity per data set; the second field abbreviates the entity, followed by the primary data reference (see Supplementary Figure S1a). The molecular regulators of apoptosis are listed on the *y* axis. (**b**) Representative sections of histological specimen from lymph node biopsies stained for MCL-1 or BCL-2 after staining for target protein and counterstained with hematoxylin. Scale bars: 100 µm. (**c**) Collective assessment of MCL-1 or BCL-2 expression determined by immunohistochemistry in histological specimen derived from three patient cohorts representing 25 PTCL-NOS, 8 ALCL and 7 AITL patients. Samples with $>50\%$ positive tumor cells were scored as 'high'.

not complete and the heterozygously deleted *Mcl-1* mice eventually succumbed to lymphoma development (Figure 2c). We found that the elevated expression of alternative antiapoptotic BCL-2 proteins such as BCL-X_L or BCL-2 conveyed resistance against the targeting of MCL-1 in the lymphoma samples of *Mcl-1*$^{fl/+}$*IS*$^{+/-}$ *CD4Cre* mice (Supplementary Figures S2c and e).

Of note, the complete genetic loss of *Mcl-1* was even more potent in reducing T-cell numbers in *Mcl-1*$^{fl/fl}$*IS*$^{+/-}$ *CD4Cre* mice at 4 and 16 weeks and further delayed lymphoma onset when compared with control mice (Figures 2a–c). However, as preclinical target exploitation was the ultimate translational incentive beyond resolving the pathogenic relevance of MCL-1, and given

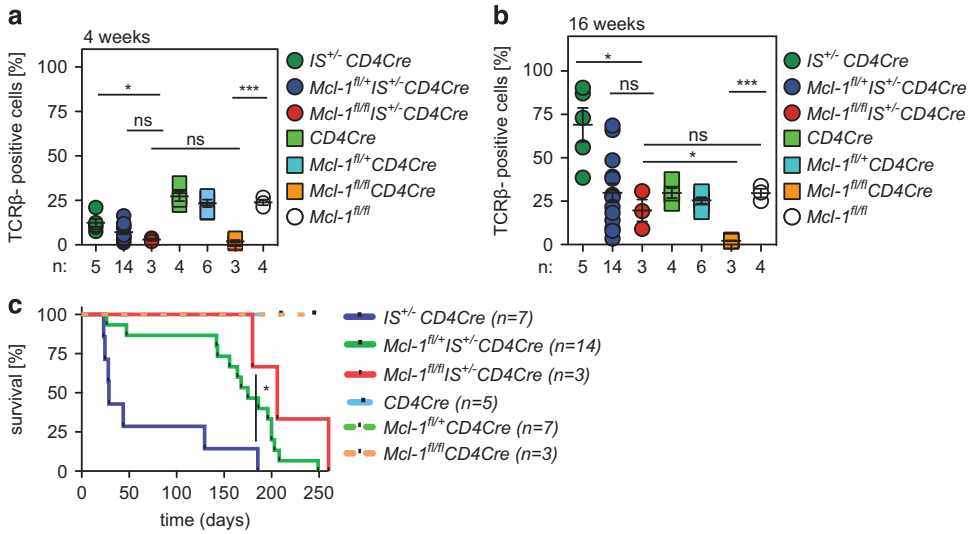

Figure 2. Deletion of one allele of *Mcl-1* delays lymphoma initiation. Flow cytometric analysis of the PB of (**a**) 4- and (**b**) 16-week-old *ITK-SYK*$^{+/-}$*CD4Cre* (*IS*$^{+/-}$*CD4Cre*) mice. Shown is the relative number of TCRβ^{+} cells in *IS*$^{+/-}$*CD4Cre* ($n=5$), *Mcl-1*$^{fl/+}$*IS*$^{+/-}$*CD4Cre* ($n=14$), *Mcl-1*$^{fl/fl}$*IS*$^{+/-}$*CD4Cre* ($n=3$), *CD4Cre* ($n=4$), *Mcl-1*$^{fl/+}$*CD4Cre* ($n=6$) and *Mcl-1*$^{fl/fl}$*CD4Cre* mice ($n=4$). Shown is the mean ± s.e.m. Statistical analysis was performed by unpaired *t*-test and asterisks denote significant differences (*$P<0.05$, ***$P<0.0005$). (**c**) Kaplan–Meier survival curves of *IS*$^{+/-}$*CD4Cre* ($n=7$), *Mcl-1*$^{fl/+}$*IS*$^{+/-}$*CD4Cre* ($n=14$), *Mcl-1*$^{fl/fl}$*IS*$^{+/-}$*CD4Cre* ($n=3$), *CD4Cre* ($n=5$), *Mcl-1*$^{fl/+}$*CD4Cre* ($n=7$) and *Mcl-1*$^{fl/fl}$*CD4Cre* ($n=3$) mice. Statistical analysis was performed by Mantle–Cox test ($P=0.001$). NS, not significant.

the expected toxicity of complete MCL-1 inhibition in a clinical setting, we primarily focused here on the characterization of partial genetic deletion of MCL-1 in *Mcl-1*$^{fl+l}$*IS*$^{+/-}$*CD4Cre* mice.

Together, these data showed that MCL-1 protected T cells during the process of malignant transformation and even a 50% reduction in MCL-1 level was sufficient to significantly delay lymphoma onset in mice.

Deletion of one allele of Mcl-1 impairs the survival of T-cell lymphoma cells

To understand the role of MCL-1 during the sustenance of T-NHL cells in fully established lymphoma, we utilized the murine T-NHL lymphoma model initially described by Kaplan.[26] Sequential low-dose γ-irradiations initiate the development of T-NHL in mice. This disease is characterized by T-NHL cell infiltration into several organs (Supplementary Figures S3a and b) and lymphoma cells usually express both CD4 and CD8 (occasionally one or neither of these markers) in addition to activation markers, such as CD69, CD44 and CD25 (Supplementary Figure S3c). Based on clinical parameters and the marker expression and consistent with previous publications, the T-NHL obtained in this model is considered a murine model similar to human PTCL-NOS.[27,29] Using *Mcl-1*^{floxed} *CreERT2*$^{ki/+}$ mice[33] (termed *Mcl-1*^{floxed} *CreER* mice) or *Bcl-X*$_L^{floxed}$*CreERT2*$^{ki/+}$ mice[37] (termed *Bcl-X*$_L^{floxed}$*CreER* mice), we can inducibly delete *Mcl-1* or *Bcl-X*$_L$ in the fully established disease at a given time point by tamoxifen treatment. After disease onset, the primary lymphoma cells can be characterized *ex vivo* (Figure 3) or transplanted into syngeneic and immunocompetent C57BL/6 recipients to characterize lymphoma cell survival *in vivo* (Figure 4). We harvested lymphoma cells from clinically symptomatic lymphoma-carrying *Mcl-1*$^{fl/+}$*CreER* and *Mcl-1*$^{+/+}$*CreER* mice and measured cell survival *ex vivo* after 48 h of inducing Cre activity (by treatment with tamoxifen). Deletion of one allele of *Mcl-1* resulted in a substantially elevated and specific induction of apoptosis as measured by FACS (fluorescence-activated cell sorting) of T-NHL cells (Figures 3a and b and Supplementary Figure S3d). Heterozygous loss of *Mcl-1* reduced protein levels of MCL-1 to about 50% and liberated the intrinsic apoptotic pathway from MCL-1-mediated inhibition as revealed by the appearance of processed Caspase-3 (Figure 3c). This lymphoma cell killing was prevented when MCL-1 was ectopically overexpressed in *Mcl-1*$^{\Delta/+}$ lymphoma cells (Supplementary Figures S3e and f).

To test the importance of MCL-1 to protect lymphoma cells against apoptosis induced by cytotoxic insults, we subjected them to standard T-NHL chemotherapeutics. We observed almost complete killing of all cultured *Mcl-1*$^{\Delta/+}$ lymphoma cells treated with doxorubicine, cyclophosphamide or etoposide, whereas control cells survived at significantly higher rates (Figure 3d and Supplementary Figure S3g).

We next sought to corroborate the role of MCL-1 in human T-cell lymphoma cell lines. We cultured the widely used mature T-cell leukemia/lymphoma cell lines Hut78 and MyLa, which originate from patients with cutaneous T-cell leukemia/lymphoma. For silencing of *MCL-1*, we constructed short hairpin RNA (shRNA)-encoding lentiviral vectors to specifically downregulate *MCL-1* expression. After transduction and subsequent FACS of gene-silenced cells, quantification of protein levels revealed a markedly reduced MCL-1 expression in both cell lines (Figure 3f). Accordingly, the specific knockdown of *MCL-1* inhibited cellular growth in both analyzed human T-cell lines when directly compared with control-shRNA treatment (Figure 3g). In addition, inhibition of MCL-1 by the compound UMI-77, which has been reported to possess activity for binding and inhibiting MCL-1 within its BH3 groove,[46] showed a specific and dose-dependent killing activity in these cell lines (Supplementary Figure S3i)

Together, MCL-1 'haploinsufficiency', based on partial genetic loss or shRNA-mediated silencing of *Mcl-1*, substantially impaired the continued survival of established human and mouse T-cell lymphoma cells.

Minimal protection against apoptosis afforded by the alternative BCL-2 family members BCL-2, BCL-X$_L$ and BCL-W

BCL-X$_L$ potently protects MYC-driven lymphoma of B-cell origin against apoptosis during lymphomagenesis.[30] We hypothesized that additional pro-survival BCL-2 family members such as BCL-X$_L$, BCL-2 or BCL-W might cooperate with MCL-1 to protect T-NHL cells. We tested the killing potential of the BH3-mimetic compound ABT-737, which binds and inhibits BCL-2, BCL-X$_L$ and BCL-W but not MCL-1 or A1.[47] We observed no effect of ABT-737 as a single agent and only marginally increased apoptosis by

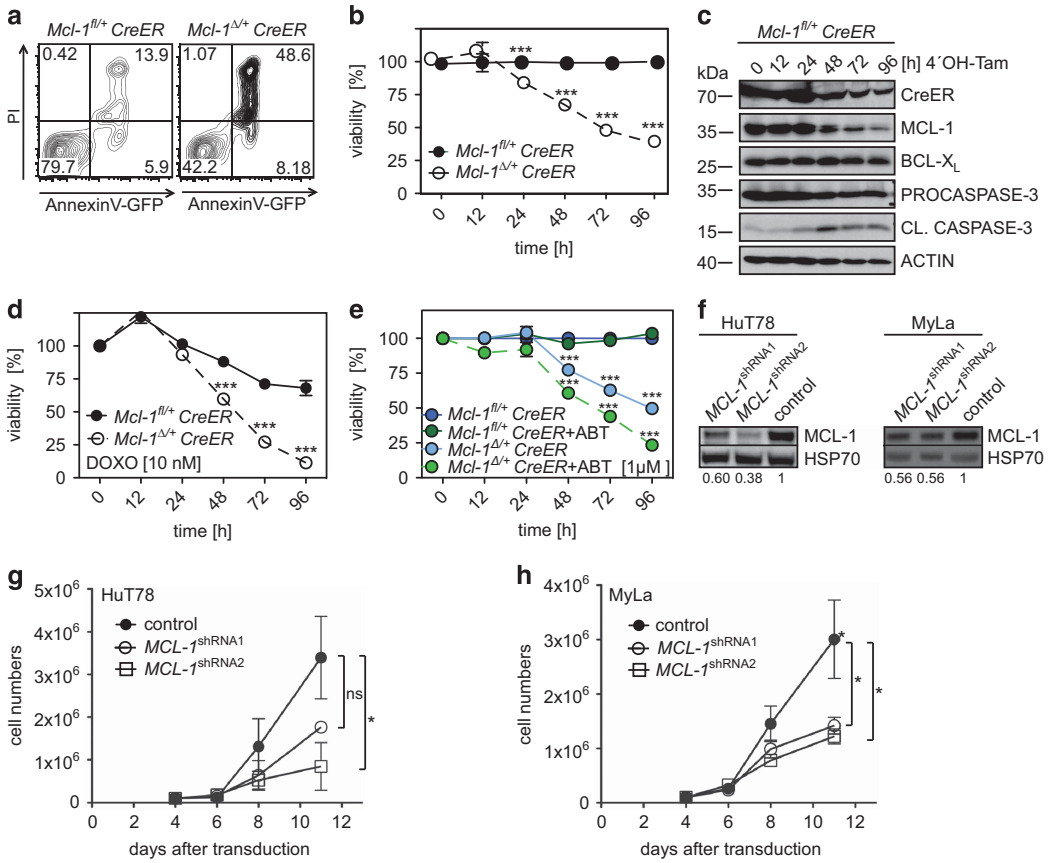

Figure 3. Induced deletion of one allele of *Mcl-1* potently induces cell death and sensitizes T-NHL cells to chemotherapy. (**a**) The *in vitro* survival of T-NHL cells from diseased *Mcl-1*$^{fl/+}$*CreER* mice treated with either 100 nM 4´OH-Tam (to delete *Mcl-1*^{fl}) or EtOH (vehicle) was followed for 48 h. (**b**) The survival of T-NHL cells from diseased *Mcl-1*$^{fl/+}$*CreER* mice treated with either 100 nM 4´OH-Tam (dashed line) or EtOH (continuous line) was tested for the indicated time points. (**c**) Immunoblotting of *Mcl-1*$^{fl/+}$*CreER* cells after treatment with 4´OH-Tam for the indicated time points. (**d**) Viability of *Mcl-1*$^{fl/+}$*CreER* T-NHL cells that had been treated with 100 nM 4´OH-Tam (dashed line) or vehicle (continuous line) and co-treated with 10 nM doxorubicine (DOXO). Cell survival was measured at the indicated time points by using the Cell Titer Glo Luminescent cell viability assay. (**e**) The survival of *Mcl-1*$^{fl/+}$*CreER* T-NHL cells that had been treated with 100 nM 4´OH-Tam (bright green and blue) or vehicle (dark green and blue) and co-treated with ABT-737 at (1 μM; green lines) or vehicle (blue lines) for the indicated time points. Cell survival was examined by using the Cell Titer Glo Luminenscent Assay. All experiments were carried out three times with three replicates each and data represent the mean ± s.e.m. Asterisks denote significant differences (*$P < 0.05$, ***$P < 0.0005$). Statistical analysis was performed by unpaired *t*-test. (**f**) Immunoblot of human T-cell lymphoma cell lines HuT78 and MyLa 4 days after lentiviral transduction with MCL-1-specific shRNA#1 (open circle) or shRNA#2 (open square) or with a scrambled, unspecific control shRNA (full circle). Numbers below blots indicate control (HSP70) normalized densitometric quantitation of MCL-1 bands, 1 is corresponding to the level in the control-shRNA treated cells. (**g** and **h**) Cell numbers of sorted human T-cell lymphoma cell lines (**g**) HuT78 and (**h**) MyLa transduced as described in panel (**f**) and monitored over 7 days. Data represent triplicates depicted as the mean ± s.e.m. Asterisks denote significant differences (*$P < 0.05$). Statistical analysis was performed by unpaired *t*-test. NS, not significant.

ABT-737 over the level achieved by partial genetic deletion of MCL-1 in T-NHL cells (Figure 3e and Supplementary Figure S3h). This implies that MCL-1 and potentially BFL-1/A1, but none of the other antiapoptotic BCL-2 family members, is responsible and required for lymphoma cell survival.

We substantiated this finding using *Bcl-x*$_L^{floxed}$ (termed *Bcl-x*$_L^{fl}$) mice in the irradiation-based murine T-NHL model, allowing for the inducible deletion of *Bcl-x*$_L$ at any given time point either by transduction with a Cre-expressing construct or by expressing CreER as a transgene from the Rosa26 locus. We found that only the complete genetic deletion of *Bcl-x*$_L$ moderately affected lymphoma cell survival *ex vivo* (Supplementary Figure S3j). Despite the complete loss of BCL-X$_L$ protein levels observed by immunoblotting 96 h after Cre-mediated deletion of *Bcl-x*$_L^{fl}$ (termed *Bcl-x*$_L^{\Delta/\Delta}$*CreER*) (Supplementary Figure S3k), apoptosis was only slightly increased (Supplementary Figure S3j), which was prevented by exogenous overexpression of BCL-X$_L$ (Supplementary Figure S3j). In contrast to the results obtained in *Mcl-1*-targeted cells, apoptosis induction in response to the genetic deletion of *Bcl-x*$_L$ became apparent only after the complete homozygous deletion but not after partial deletion of *Bcl-x*$_L$ (Supplementary Figures S3l and m). Moreover, combinatorial treatment of *Bcl-x*$_L$ fully deleted cells with doxorubicine induced only modest toxicity (Supplementary Figure S3n).

These results showed that MCL-1 and possibly BFL-1/A1 but none of the alternative BCL-2 proteins is the critical antiapoptotic BCL-2 family member for the continued survival of T-NHL cells in this model.

Heterozygous deletion of Mcl-1 delays lymphoma engraftment in vivo

To test the functional relevance of MCL-1 for the sustained survival of T-NHL cells *in vivo*, we transplanted *Mcl-1*$^{fl/+}$*CreER* lymphoma cells into syngeneic and immunocompetent wild-type C57BL/6-recipient mice. At 16 days after injection, a time point when the lymphomas were fully established in the recipients, tamoxifen or vehicle was given in a therapeutical approach by oral gavage to activate CRE and induce the deletion of one allele of *Mcl-1* in the lymphoma cells (Supplementary Figure S4a).

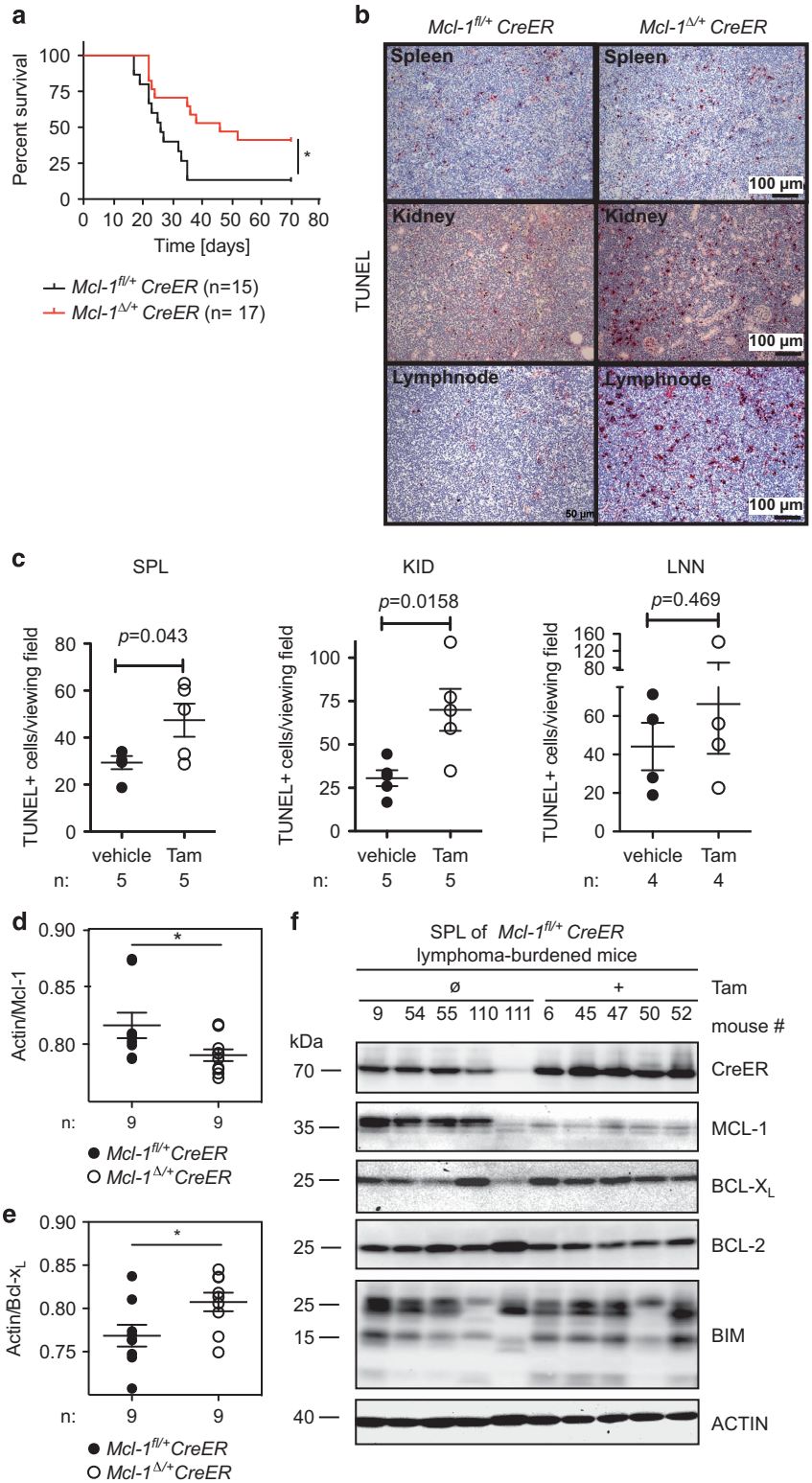

Figure 4. Loss of one allele of *Mcl-1* delays lymphoma expansion *in vivo*. (**a**) Kaplan–Meier survival curves of mice that received *Mcl-1*$^{fl/+}$*CreER* T-NHL cells and were treated either with vehicle (black line; $n=15$; median survival 26 days) or tamoxifen (to delete *Mcl-1*^{fl}; red line; $n=17$; median survival 46 days). Statistical analysis was performed by Mantle–Cox test ($P=0.012$). (**b**) Histological analysis of cell death induction using TUNEL staining (red) in the indicated organs of mice transplanted with *Mcl-1*$^{fl/+}$ *CreER* T-NHL cells 48 h after tamoxifen (*Mcl-1*$^{\Delta/+}$ *CreER*) (right) or vehicle treatment (*Mcl-1*$^{fl/+}$ *CreER*) (left). The scale bar indicates 100 µm. (**c**) Infiltration of apoptotic cells (TUNEL+) in the depicted organs of mice treated as in panel (**b**) as determined by quantification of TUNEL staining per viewing field (five mice per condition for spleen and kidney; four mice per condition for lymph node histology). Statistical analysis was performed by unpaired *t*-test. (**d** and **e**) Quantitative PCR analysis of mRNA expression of (**d**) *Mcl-1* ($P=0.0424$) or (**e**) *Bcl-x*$_L$ ($P=0.0349$) in T-NHL cells of sick mice that had received vehicle or tamoxifen treatment. Statistical analysis was performed by unpaired *t*-test. (**f**) Immunoblotting analysis of T-NHL cells of sick control mice (mouse nos. 9, 54, 55, 110, 111) and mice that had received tamoxifen (mouse nos. 6, 45, 47, 50, 52). Probing for ACTIN served as loading control. *$P=0.05$.

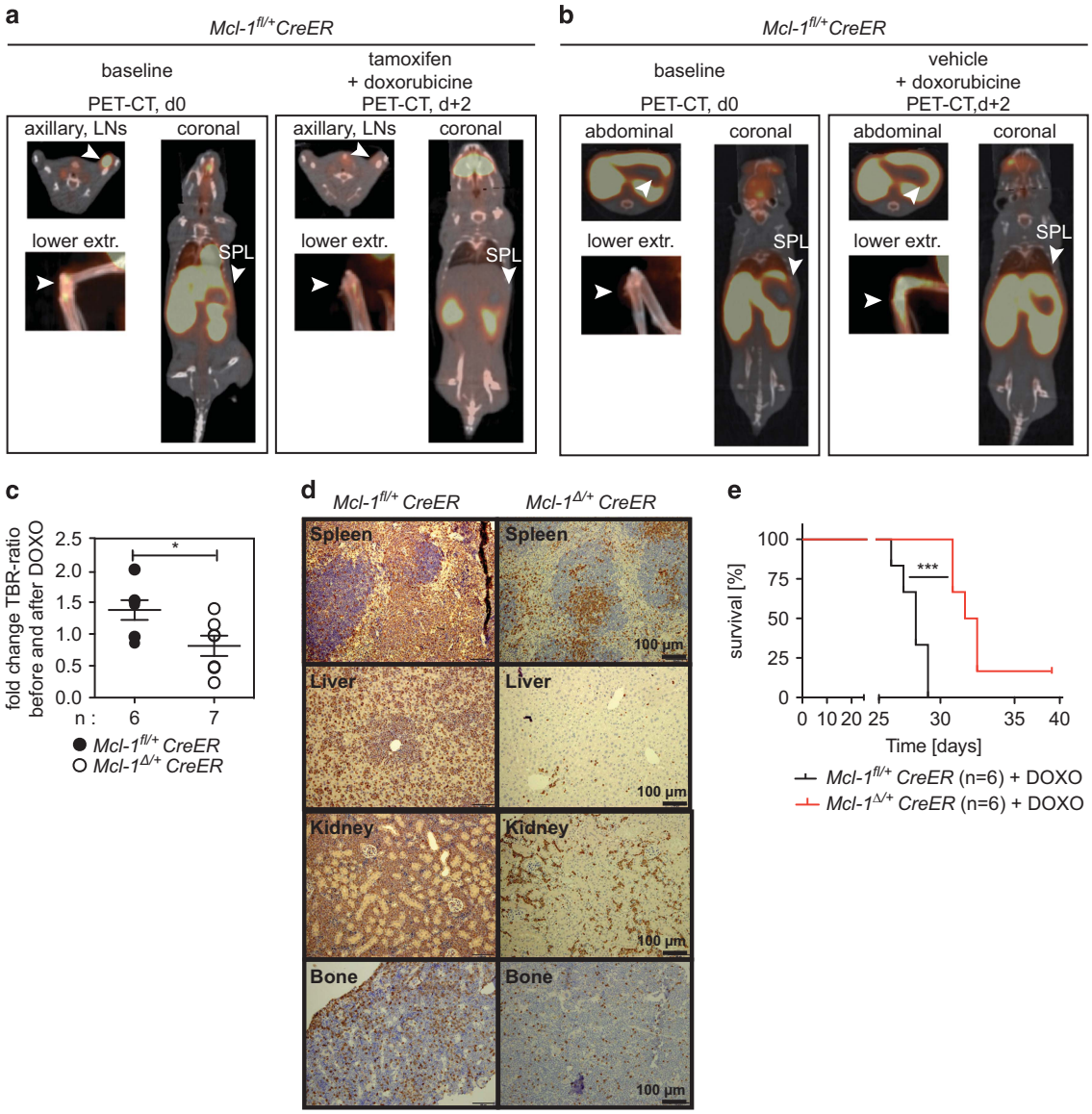

Figure 5. *Mcl-1* deletion and chemotherapeutic drug treatment synergize in killing lymphoma cells *in vivo*. (**a**) PET-CT images of mice transplanted with 1×10^7 *CreER Mcl-1*$^{fl/+}$ T-NHL cells after treatment with doxorubicine and co-treatment with tamoxifen. White arrows mark regions of interest. (**b**) PET-CT of a mouse transplanted with 1×10^7 *CreER Mcl-1*$^{fl/+}$ T-NHL cells after treatment with doxorubicine alone. White arrows mark regions of interest. (**c**) Fold change of ^{18}F-FDG uptake estimated as tumor to background ratio (TBR) before and after doxorubicine treatment with ($n = 7$) or without ($n = 6$) tamoxifen treatment. Statistical analysis was performed by unpaired *t*-test ($P = 0.029$). $^*P < 0.05$, $^{***}P < 0.0005$. (**d**) Histological analysis of T lymphoma cell infiltration by staining for TCRβ in the indicated organs of mice transplanted with *Mcl-1*$^{fl/+}$ *CreER* T-NHL cells after doxorubicine treatment. Depicted are representative sections of vehicle-treated control mice (left panel) and tamoxifen-treated mice (right panel). The scale bar indicates 100 µm. (**e**) Kaplan–Meier survival curves of mice that received 1×10^7 *Mcl-1*$^{fl/+}$ CreER T-NHL cells and treated with 4 mg/kg doxorubicine on day 17. The red line depicts mice that had also received tamoxifen (4 mg) on days 16 and 17 ($n = 6$; median survival 32.5 days) and the black line shows mice that had received doxorubicine only ($n = 6$; 28 days). Statistical analysis was performed by Mantle–Cox test ($P = 0.0008$).

Consistent with a critical survival function of MCL-1, we observed that the deletion of a single allele of *Mcl-1* resulted in a significantly prolonged survival of lymphoma-bearing mice when compared with vehicle-treated mice (Figure 4a), which occurred irrespective of toxicity by aberrant CRE as shown in the respective control mice (Supplementary Figure S4b). Genetic deletion of a single allele of MCL-1 in lymphoma cells by tamoxifen treatment also resulted in significantly elevated numbers of apoptotic cells in various organs of lymphoma-burdened mice when compared with vehicle controls (Figures 4b and c). These data are consistent with the critical survival function of MCL-1 observed in lymphoma cells *ex vivo* and support the notion that the protection against apoptotic cell death by MCL-1 is a critical prerequisite for the survival and expansion of T-NHL cells *in vivo*.

We next tested whether the recombination event in *Mcl-1*$^{fl/+}$ *CreER* lymphoma cells was efficient and whether incomplete deletion might have served as a resistance mechanism. Deletion of *Mcl-1*^{fl} was efficiently achieved upon tamoxifen treatment in *Mcl-1*$^{fl/+}$ *CreER* lymphoma cells *in vivo* as demonstrated by analysis of genomic DNA, RNA and protein (Figures 4d and f and Supplementary Figures S4c and d). Similar to the elevated expression of alternative antiapoptotic BCL-2 family members seen in the ITK-SYK T-NHL model upon *Mcl-1* deletion (Figure 2),

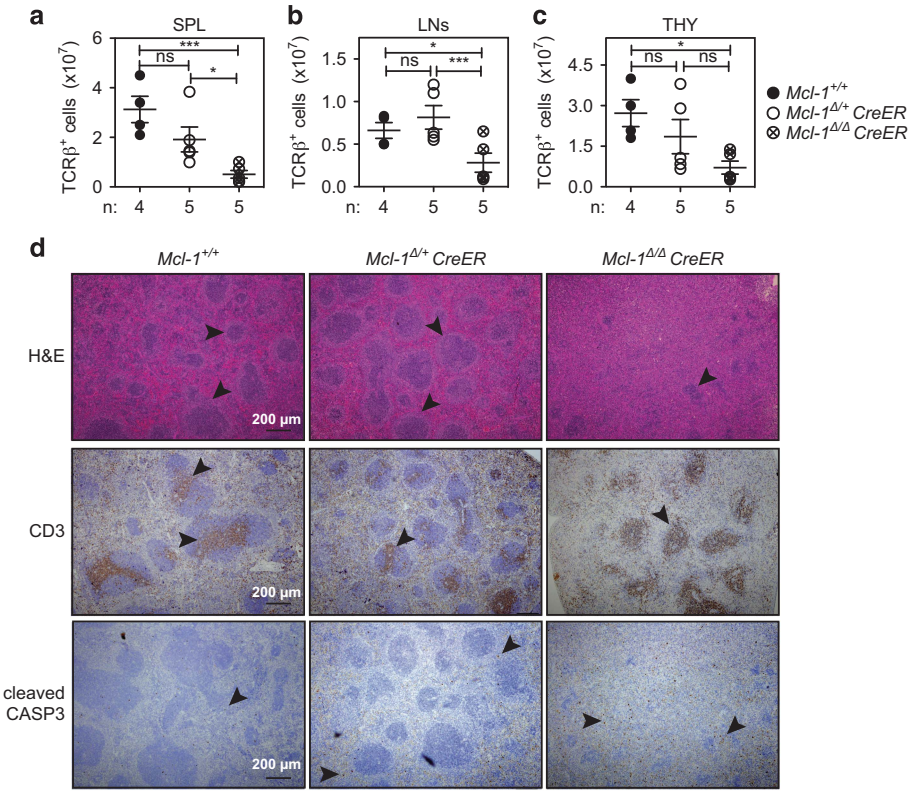

Figure 6. Minimal impact of the induced loss of one allele of *Mcl-1* on the survival of normal T lymphocytes. (**a**) Number of T cells in the spleens of mice with and without *Mcl-1* deletion. Mice of the respective genotypes were treated by oral gavage with tamoxifen (to delete *Mcl-1*^{fl}; 4 mg) for 2 days. Mice were killed on day 3 and the organs were isolated. Splenocytes were stained for TCRβ expression and analyzed by flow cytometry. Statistical analysis was performed by unpaired *t*-test (p*Mcl-1*$^{+/+}$ vs $^{\Delta/+}$ = 0.14; p*Mcl-1*$^{+/+}$ vs $^{\Delta/\Delta}$ = 0.012; p*Mcl-1*$^{\Delta/+}$ vs $^{\Delta/\Delta}$ = 0.03). (**b**) The numbers of T cells in the lymph nodes of mice treated as in panel (**a**) (p*Mcl-1*$^{+/+}$ vs $^{\Delta/+}$ = 0.4; p*Mcl-1*$^{+/+}$ vs $^{\Delta/\Delta}$ = 0.04; p*Mcl-1*$^{\Delta/+}$ vs $^{\Delta/\Delta}$ = 0.02). (**c**) The numbers of T cells in the thymus of mice treated as in panel (**a**) (p*Mcl-1*$^{+/+}$ vs $^{\Delta/+}$ = 0.006; p*Mcl-1*$^{+/+}$ vs $^{\Delta/\Delta}$ = 0.33; p*Mcl-1*$^{\Delta/+}$ vs $^{\Delta/\Delta}$ = 0.13). *$P < 0.05$, ***$P < 0.0005$. (**d**) Histological analysis of the spleens after *Mcl-1* deletion. Shown are sections of the spleens of *Mcl-1*$^{+/+}$, *Mcl-1*$^{fl/+}$ *CreER* (*Mcl-1*$^{\Delta/+}$) or *Mcl-1*$^{fl/fl}$ *CreER* (*Mcl-1*$^{\Delta/\Delta}$) mice that had been treated by oral gavage with tamoxifen (4 mg) for 2 days. The first panel shows hematoxylin (blue) and eosin (pink) staining, the second panel shows CD3 expression (brown) and the third panel shows cleaved CASPASE 3 (cleaved Casp 3; brown). The black arrows show examples for positively stained cells. Depicted is one representative picture for mice of each genotype. Scale bars represent 200 µm. NS, not significant.

we observed elevated mRNA and protein levels of BCL-X_L, but not of BCL-2, in the lymphoma cells following genetic deletion of *Mcl-1*, which might represent a mechanism of compensation for the loss of MCL-1 in T-NHL cells (Figures 4e and f).

These data showed that the survival of fully established T-NHL required the continued expression of MCL-1 and that even partial loss of *Mcl-1* sufficed to extend survival of lymphoma-burdened mice.

Impact of Mcl-1 deletion and doxorubicine treatment on T lymphoma cells in vivo

We observed a strong sensitization of lymphoma cells to conventional CHOP-based chemotherapeutics upon deletion of one allele of *Mcl-1 in vitro* (Figure 3d and Supplementary Figures S3g and h). To test this effect *in vivo*, we generated cohorts of wild-type syngeneic immunocompetent recipient mice injected with *Mcl-1*$^{fl/+}$ *CreER* or control lymphoma cells. We allowed T-cell lymphoma to become fully established in the recipient mice over a period of 16 days before treatment with doxorubicine and tamoxifen (for deletion of one allele of *Mcl-1*) commenced (Supplementary Figure S5a). To assess lymphoma burden, we performed *in vivo* imaging of mice by ^{18}F-FDG–PET–CT (computed tomography) scan at baseline and 2 days after treatment. Consistent with a potent sensitization to doxorubicine, we observed a reduction in the lymphoma burden in mice that harbored *Mcl-1*$^{\Delta/+}$ *CreER* T-NHL cells as early as 2 days after treatment (Figure 5a). This is exemplified by the reduced ^{18}F-FDG tracer uptake in the liver, axillar lymph nodes and bone marrow (Figure 5a). In contrast, mice treated with doxorubicine alone did not show a significant reduction in tracer uptake at this time point (Figure 5b). Quantification of signal intensities showed a significant difference in tracer uptake before and after doxorubicine treatment only for mice with *Mcl-1*-deleted lymphoma cells (Figure 5c), which is also exemplified by the spleen to body weight ratio (Supplementary Figure S5b) and T-cell infiltration in the spleen (Supplementary Figure S5c) and lymph nodes (Supplementary Figure S5d) as well as in the kidney, liver and bone marrow (Supplementary Figures S5e–g).

Of note, partial genetic deletion of *Mcl-1* was sufficient to extend the lifespan of doxorubicine-treated mice when compared with mice bearing control lymphomas (Figure 5e). These findings showed that the reduction in MCL-1-sensitized lymphoma cells to conventional chemotherapeutics and extended survival of lymphoma-burdened mice.

Healthy T lymphocytes remained largely unaffected by mono-allelic Mcl-1 deletion

MCL-1 has an important role for the development and survival of normal T lymphocytes.[35] To address the question whether a therapeutic window for the inhibition of MCL-1 in T cells exists, we monitored overall performance status and lymphoid organs in *Mcl-1*^{floxed} *CreER* mice. As previously published, the complete genetic deletion of *Mcl-1* depleted the T-cell compartment of

experimental mice (Figures 6a–d).[34,35] However, to mimic the inhibition of MCL-1 afforded by a, yet to be defined, pharmacological inhibitor in the clinical setting, we tested the effects of loss of only one allele of *Mcl-1*, which afforded a reduction in protein levels to approximately 50% (Figure 3c).

We found that the mono-allelic deletion of *Mcl-1* only minimally impacted on the overall performance status of the experimental mice and only marginally affected lymphoid parameters such as spleen size (Supplementary Figure S6a) as well as T-cell numbers in the spleen (Figure 6a), lymph nodes (Figure 6b) and thymus (Figure 6c). Histological analysis of spleen sections showed normal distribution of white and red pulp and no structural or compositional abnormalities in *Mcl-1*$^{\Delta/+}$ mice, as exemplified by the amount of CD3$^+$ T cells and Caspase-3 activation in wild-type and *Mcl-1*$^{fl/+}$ *CreER* mice (Figure 6d). Moreover, 4 weeks after CRE-mediated deletion, T-cell numbers in the lymph nodes (Supplementary Figure S6c) and thymus (Supplementary Figure S6d) remained unaffected by *Mcl-1* deletion and T lymphocytes were only slightly reduced in the spleens of these mice (Supplementary Figure S6b). Importantly, experimental mice did not show any clinical symptoms during the observational period. Moreover, the reduction in CD19$^+$ B-cell numbers in the spleens and lymph nodes of these *Mcl-1* gene-targeted mice was only marginal (Supplementary Figures S6e–h). Similar results were observed for myeloid cells (Supplementary Figures S6i and j).

Together, these data showed that the heterozygous deletion of *Mcl-1* had only a minor impact on the normal T-cell compartment and mice remained clinically unaffected. MCL-1 might therefore serve as an attractive pharmacological target to re-activate programmed cell death in T-NHL cells when selective pharmacological MCL-1 inhibitors become available.

DISCUSSION

Patients diagnosed with T-cell lymphoma have a dismal prognosis.[48] This is primarily based on their often-aggressive natural course and a notorious resistance to conventional chemotherapeutic drugs. The recently generated plethora of genomic data in T-NHL has improved our definition of different T-NHL subtypes. However, in the absence of strategies that target the specific nature of the malignant T-cell, chemotherapy remains the standard therapeutic approach for most T-NHL patients. Novel molecular therapies such as anti-CD30 antibodies or ALK inhibitors have proven a successful implementation into the clinic, but their use is restricted to specific patient subgroups.

Conventional cytogenetic analysis, comparative genomic hybridization and single-nucleotide polymorphism array studies of considerable cohorts of human T-NHL patients have revealed that defined recurrent genetic aberrations in T-NHL are rare. Instead, most patients carry multiple genomic imbalances or complex karyotypes.[21] This identifies T-NHL as a genetically heterogeneous group of lymphoid malignancies and underscores the need to identify common molecular vulnerabilities that might be targeted for T-NHL therapy in a majority of T-NHL patients.

Using publicly available gene expression profiling data sets, we found high *MCL-1* expression to be shared by most human T-NHL subsets (Figure 1). This finding is reminiscent of the gains in the chromosomal region 1q, where MCL-1 is located, which was observed in PTCL-NOS and ALK-negative ALCL patient samples[16,49,50] as well as the gains of the *MCL-1* gene locus associated with more aggressive subtypes of cutaneous T-cell lymphoma[1,3,7] and adult T-cell leukemia/lymphoma.[1,51]

Of note, the *MCL-1* mRNA expression translated into prominent protein abundance in most T-NHL patient samples tested. This is consistent with the finding that MCL-1 was the most abundant antiapoptotic BCL-2 family member in a histological study on various different human peripheral T-NHL samples[1,16,52] and on skin biopsies from patients with Mycosis fungoides or Sezary Syndrome.[2–13,18–22,53] In light of its high turnover rate and short half-life,[21,54] the high levels of MCL-1 protein observed in the patient samples further support the notion that the continued protection against apoptotic cell death by MCL-1 is a critical hallmark of T-NHL. This is further underscored by the finding that ALCL patients often present with a reduction in miR29a expression, which is known to repress *Mcl-1* RNA. This repression of miR29a, likely caused by aberrant methylation, results in MCL-1 overexpression and effective apoptotic blockade in this T-NHL subtype.[21,25,55]

Our analysis of the gene expression data also identified high expression of A1/BFL-1 (A1-001 and A1-002) in several T-NHL subtypes, such as AITL[5,24,56] and ALCL.[20,24] A1/BFL-1 has a similar binding profile for BH3-only proteins and BAK/BAX as MCL-1 and elevated A1/BFL-1 expression has been suggested as a resistance mechanism against chemotherapy and treatment with BCL-2/BCL-X_L-specific BH3 mimetic drugs similar to what was described for MCL-1.[24,28,30–32,57] Functional studies on the role of A1/BFL-1 have been hampered thus far by the difficulty to generate conventional gene-targeted mouse model systems and the lack of pharmacological inhibitors for A1/BFL-1.

Loss of BCL-X_L had very little impact on the survival of fully established T-NHL cells in our model system. This finding is consistent with its low mRNA expression level in human T-NHL specimens and reminiscent of the finding that loss of BCL-X_L had no impact on T-cell lymphomagenesis in *p53*$^{-/-}$ mice.[34–36,44] In addition, we observed only a minimal effect of pharmacological inhibition of BCL-2, BCL-X_L and BCL-W using the BH3-mimetic compound ABT-737 on T-NHL cells. These findings clearly support the notion that MCL-1 (and perhaps A1/BFL-1), but none of the other antiapoptotic BCL-2 family members, controls T-NHL survival.

Using two independent mouse models of T-cell lymphomagenesis, we found that MCL-1 controls both T-NHL development and the continued T-NHL survival *in vivo*. The significantly delayed lymphoma onset in ITK-SYK-expressing mice after *Mcl-1* deletion argues that MCL-1 protects nascent neoplastic T lymphoid cells from oncogenic, hence pro-apoptotic stress, that is present during malignant transformation.

Using the irradiation-based T-NHL mouse model, we can mimic the clinical situation by targeted deletion of one or both alleles of MCL-1 after disease onset in lymphoma-burdened mice. Here we observed a significantly delayed lymphoma reconstitution after loss of one allele of *Mcl-1*. A similar exquisite dependence on MCL-1 has also been described in MYC-driven AML,[38,58,59] MYC-driven B-NHL,[31,39] BCR-ABL-driven pre-B-acute lymphoblastic leukemia[41,42,60] and in p53-mutated thymic lymphoma.[44] In these entities, loss of even a single allele of MCL-1 was sufficient to induce apoptosis and to promote disease regression. These findings support the notion that pharmacological targeting of MCL-1 might be a powerful tool to induce apoptosis in T-NHL cells once specific drugs become available.[26,61]

The characterization of systemic toxicity of MCL-1 deletion will be critical to allow for MCL-1 inhibitors to transition into the clinical setting. Previous publications have shown that the complete genetic deletion of MCL-1 in all cells from conception causes early embryonic lethality[27,62] and complete loss of MCL-1 in selected cell types (using tissue-specific CRE strains) depleted hematopoietic stem/progenitor cells,[44,63] B cells and T cells,[2–13,18–20,34] memory B cells,[33,64] plasma cells[37,65] and cardiomyocytes.[33,66,67]

However, the loss of only a single allele of *Mcl-1*, which reduced MCL-1 protein levels in T-NHL cells to about 50% (Figures 3c and 4d and Supplementary Figure 2e), was sufficient to kill malignant T cells and significantly extended the survival of lymphoma-burdened mice. This suggests that it may not be necessary to completely inactivate MCL-1 to achieve a clinical benefit. Importantly, we and others observed only minimal toxic effects on healthy T lymphocytes, spleen size and on the overall performance status

of experimental mice after loss of 50% MCL-1 protein over a period of 4 weeks (Figure 6 and Supplementary Figure 6).[23,31,34,44,57,58] Consistent with this, it was recently reported that *Mcl-1*$^{+/-}$ mice have normal appearance and hematopoietic cell subset numbers in the absence of stress.[44,68]

Collectively, this argues that the cytotoxic stresses experienced by T-NHL cells might render them highly dependent on MCL-1 and thus offer a therapeutic window for the targeting of MCL-1, either alone or in combination with conventional chemotherapy. It is, however, noteworthy that constitutive loss of one allele of *Mcl-1* rendered the hematopoietic stem/progenitor cells highly sensitive to DNA damage.[34,44] Thus care will need to be taken on how to schedule combinatorial treatments with MCL-1 inhibitors plus chemotherapeutic drugs.

In conclusion, our data argue that T-NHL cells are exquisitely dependent on the pro-survival BCL-2 family member MCL-1 for their survival and that patients diagnosed with T-cell lymphoma might profit from pharmacological approaches that inhibit MCL-1 function irrespective of the T-NHL subtype.

CONFLICT OF INTEREST

GLK and AS work at the Walter and Eliza Hall Institute. This Institute receives milestone payments from Genentech Inc. and AbbVie for the development of BH3 mimetic drugs for cancer therapy. All the other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Sabine Spinner performed experiments and wrote the manuscript. Ji-Hee Yi, Tim Schrader, Henriette Bendz, Petra Mayer, Ulrike Höckendorf, Nicole Müller and Monica Yabal performed experiments. Gemma L Kelly and Andreas Strasser generated the *CreERT2 Mcl-1*$^{fl/+}$ and *CreERT2 Mcl-1*$^{+/+}$ lymphoma cells. Enkhtsetseg Munkhbaatar performed histological analysis of murine T-NHL samples. Giuliano Crispatzu and Marco Herling integrated and summarized the mRNA expression data on human T-NHL samples. Sylvia Hartman and Martin-Leo Hansmann provided and stained histological sections of human PTCL, ALCL and AITL samples. Zoulei Lei evaluated PET-CT results on mice transplanted with T-NHL cells. Konstanze Pechloff and Jürgen Ruland generated ITK-SYK CD4Cre mice and provided critical reagents. Mathias Heikenwälder and Sebastian Newrzela provided critical reagents and advice. Christian Peschel, Andreas Strasser, Marco Herling, Ulrich Keller and Jürgen Ruland supported the project by critical discussion. Philipp J Jost designed and supervised the project and wrote the manuscript.

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