Chronic lymphocytic leukemia development is accelerated in mice with deficiency of the pro-apoptotic regulator NOXA

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Supplementary Methods

Animals

Heterozygous Eμ-TCL1 (TCL1) transgenic mice were crossed with homozygous *Noxa*$^{-/-}$ mice and housed under specific pathogen-free conditions. TCL1 mice have been backcrossed more than 10 generations onto the C57BL6(J) background. All animal experiments were approved by the animal ethical committee of the Academic Medical Center, University of Amsterdam (DSK102031).

Antibodies and flow cytometry

Samples were subjected to erythrocyte lysis and cells were subsequently stained using fluorochrome-coupled antibodies specific for CD19, CD5, CD3, B220, CD43, and CD11b (eBioscience, San Diego, CA, USA). Cells were analyzed using a FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA) and the FlowJo software (TreeStar, Ashland, OR, USA). Intracellular staining for MCL-1 was performed with the BD Cytofix/Cytoperm kit. Anti-MCL-1 was obtained from Rockland Immunochemicals Inc. (Limerick, PA, USA). Intracellular staining for Ki67 was performed using anti-Ki67 from eBioscience, and the cells were fixed and permeabilized using intracellular fixation and permeabilization reagents obtained from eBioscience.

Histology and immunofluorescence

Tissue samples were fixed in neutral buffered formalin for at least 48 hours before paraffin embedding. Sections were dewaxed and rehydrated before antigen retrieval which was done by heating the sections to 95°C for 20 minutes in citrate buffer. Subsequently, the sections were stained with anti-cleaved caspase-3 (Cell Signaling, Danvers, MA, USA) or anti-Ki67 (eBioscience).

Isolation of CLL cells from peripheral blood and spleen

Peripheral blood was obtained by cardiac puncture, and splenocytes were obtained by mashing the spleen through a 100 μm cell strainer. Samples were then subjected to erythrocyte lysis using ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) on ice. Following this, $CD5^+CD19^+$ cells were isolated from different tissues using a CD3 depletion, CD5 enrichment strategy with corresponding antibodies that were conjugated to magnetic beads (Miltenyi, Bergisch Gladbach, Germany). Samples were kept at 4ºC during the procedure. $CD5^+CD19^+$ percentage was assessed by flow cytometry and was consistently at least 90%.

RNA isolation and qPCR

RNA from $CD5^+CD19^+$ cells was isolated with RNeasy kit from Qiagen (Venlo, the Netherlands). When cell numbers were lower than 1 million, the RNeasy micro kit from Qiagen was used instead. The RNA was then converted to cDNA using Revertaid (Thermo Fisher Scientific, Waltham, MA, USA), which was subsequently used as input for qPCR, SYBRgreen was used for detection (Thermo Fisher Scientific). The following primers were used: NOXA (forward primer: 5'-ATGCCTGGGAAGTCGCAAAAGAGC-3', reverse primer: 5'-AGCACACTCGTCCTTCAAGTCTGC-3'), and ubiquitin (forward primer: 5'-TGGCTATTAATTATTCGGTCTGCAT-3', reverse primer: 5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'). The StepOnePlus Real-Time PCR system was used to analyze the samples (Thermo Fisher Scientific).

MLPA analysis

RNA from CD5+CD19+ cells from *Noxa*$^{-/-}$/TCL1 and TCL1 mice was isolated using the RNeasy kit from Qiagen and analyzed using MLPA (MRC Holland, Amsterdam, the Netherlands) which allows for gene expression analysis of a large amount of genes in a single reaction. For this analysis we used the mouse apoptosis kit to analyze apoptosis-related genes.

Clonality analysis and spectratyping of B cell populations

Total RNA was extracted from paraffin embedded spleen tissue using the RNeasy FFPE isolation kit from QIAGEN. V_H-DJ_H gene rearrangements from B cell populations were amplified using PCR primers specific for the J558 V_H region gene together with a primer specific for the Cμ constant region gene. Using a FAM-conjugated Cμ constant region or a J_H gene-specific primer in a run-off reaction, PCR products were labeled and subsequently analyzed on a capillary sequencer (ABI3100; Applied Biosystems, Leusden, NL) by fragment-length analysis. Sequences of primers were published earlier(1).

Statistical analyses

Differences in means were tested with student's T-test or, in the case of multiple comparisons, with a one-way ANOVA with Bonferroni's post hoc test. Survival was analyzed using the log-rank test.

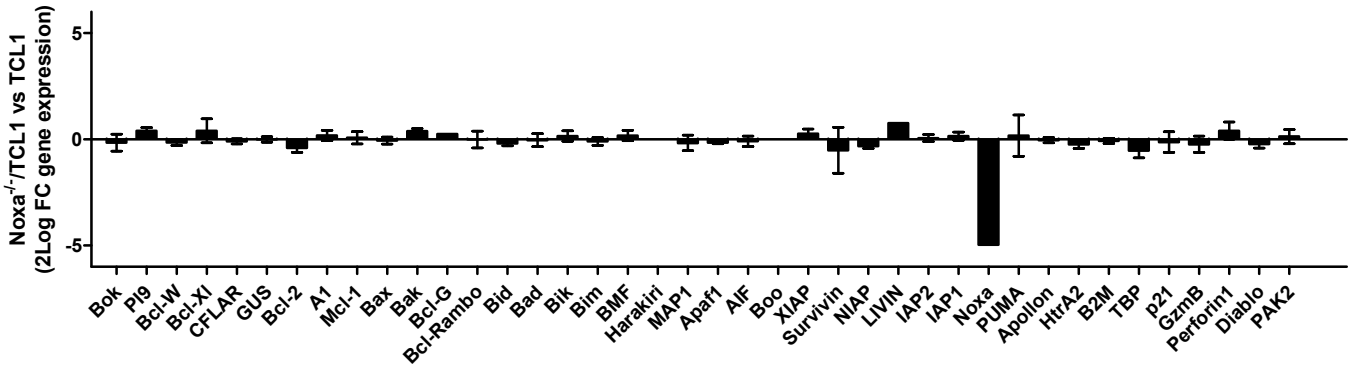
Reference list

1. Duy C, Yu JJ, Nahar R, et al. BCL6 is critical for the development of a diverse primary B cell repertoire. The Journal of experimental medicine. 2010;207(6):1209-21.

Supplemental Figure 1. Analysis of gene expression by MLPA of *Noxa*$^{-/-}$/TCL1 and TCL1 splenocytes. RNA from sorted CD5^{+}CD19^{+}cells from both *Noxa*$^{-/-}$/TCL1 and TCL1 mice was analyzed for the expression of indicated genes. Gene expression is shown as 2Log fold change between *Noxa*$^{-/-}$/TCL1 and TCL1.

Supplemental Figure 2. *Noxa*$^{-/-}$/TCL1 show decreased CLL-free survival
Survival analysis showing CLL-free survival in *Noxa*$^{-/-}$/TCL1 and TCL1 mice. Mice were considered to have developed CLL when the CD5+/CD19+ percentage in the peripheral blood exceeded 20%. Median CLL-free survival was 195 and 240 days for *Noxa*$^{-/-}$/TCL1 and TCL1 mice, respectively ($p<0.05$).

Supplemental Figure 1



Supplemental Figure 2