**IκB-ζ controls the constitutive NF-κB target gene network and survival of ABC DLBCL**

**Supplemental Material and Methods**

**Gene expression profiling and signature analyses**

Gene expression profiling was performed 24, 48, 72, and 96 hours after induction of IκB-ζ shRNA #1 in HBL-1 cells. Gene expression changes were measured in two independent biological replicates for each timepoint. Gene expression changes in HBL-1 cells in which IκB-ζ shRNA #1 was induced with doxycycline were compared to uninduced cells. The independent measurements were preprocessed and normalized in the following manner. Data were imported on raw bead level and subsequently a bead level spotfilter was applied to each microarray experiment based on the fitted density mode for the background intensities. Afterwards, bead intensities of all measured microarrays were quantile-normalized and beads were grouped by measured sequence to form beadsets. For genes having more than one measured sequence, additional merged beadsets were created. Beadsets with more than 50% of their beads excluded by the spot filter were also excluded. Further analyses were performed on gene level using median aggregation and manufacturer’s annotations.

Differentially expressed genes were identified in the following manner. A one-tailed paired t-test was used to calculate p-values for every gene based on the 8 microarray pairs. Additionally, we used the Benjamini & Hochberg method to calculate a false discovery rate (FDR) for every significance threshold. We identified 136 genes that were significantly downregulated (p<0.0025; FDR<0.09) and 88
genes that were upregulated (p<0.0025; FDR<0.09) across all timepoints following IκB-ζ knockdown (paired t-test based on two independent replicates; Supplemental Table 1).

Gene expression profiling was performed in the same manner in HBL-1 cells treated with the IKKβ inhibitor MLN120b (two independent replicates for each timepoint). Gene expression changes in HBL-1 cells that were treated with MLN120b were compared to cells that were treated with DMSO. We identified 84 genes that were significantly downregulated (p<0.0025; FDR<0.11) and 127 genes that were upregulated (p<0.0025; FDR<0.11) (Supplemental Figure 4A and Supplemental Table 2).

At last, we determined gene expression changes in HBL-1 cells 6, 12, 24, and 48 hours following treatment with the MALT1 inhibitor z-VRPR-fmk. Gene expression profiling was performed using whole-genome Agilent 4 x 44K gene expression arrays (Agilent Technologies). A “MALT1 downregulation” signature was defined as those genes that decreased in expression by at least 50% in at least 2 of the 4 timepoints (Supplemental Figure 4B and Supplemental Table 3). The “MALT1 upregulation” signature was defined as those genes that increased in expression by at least 200% in at least 2 of the 4 timepoints (Supplemental Table 3).

To obtain a better understanding of the gene expression changes, we performed an unbiased gene set enrichment analysis (GSEA) for the IκB-ζ experiment as described, using a previously curated gene expression signature database. This signature database was complemented by two signatures that we defined by treatment of HBL-1 cells with specific inhibitors against IKKβ (MLN120b) and MALT1 (z-VRPR-fmk) (see above). Signatures with less than 10 genes were excluded from this analysis (Supplemental Table 4).
Finally, we analyzed the relative \( \kappa B-\zeta \) mRNA expression between different lymphoma subtypes. Gene expression data from multiple myeloma (MM), classical Hodgkin lymphoma (cHL), Burkitt lymphoma (BL), ABC and GCB DLBCL patient samples were obtained from GEO (GSE2658, GSE12453, GSE14879, GSE40160, and GSE4732). To take considerable differences in brightness distributions of these cohorts into account, we quantile-normalized each microarray experiment based on all identical Affymetrix probesets to minimize the offsets between the cohorts. To measure the mRNA expression of \( \kappa B-\zeta \) the “223218_s_at” probeset (Affymetrix) was used. All p-values of differential \( \kappa B-\zeta \) expression were based on two-sample t-tests comparing \( \kappa B-\zeta \) expression in each lymphoma subtype to \( \kappa B-\zeta \) expression detected in ABC DLBCL (Figure 5A).

Supplemental Figures

Figure S1: \( \kappa B-\zeta \) is a nuclear \( \kappa B \) family member.
\( \kappa B-\zeta \) is localized predominantly in the nucleus of ABC DLBCL cell lines as shown by separation of nuclear and cytoplasmic proteins. BCL10 is used as cytoplasmic and PARP as nuclear marker.

Figure S2: TMD8 cells are characterized by an internal \textit{NFKBIZ} deletion.
A. TMD8 cells harbor a heterozygous internal 159 basepair deletion of \textit{NFKBIZ}, affecting amino acids 33 to 85 of \( \kappa B-\zeta \) isoform 1. Arrows indicate the position of the first nucleotide of the deletion compared to the remaining wildtype allele in TMD8 cells (left panel) and the wildtype \textit{NFKBIZ} sequence in HBL-1 (right panel).
B. NF-κB reporter activity determined in 293T cells following expression of IκB-ζ wildtype or Δ33-85 mutant. The NF-κB reporter is activated to a similar degree by wildtype and mutant IκB-ζ. Error bars indicate the standard deviation.

C. Expression of IκB-ζ wildtype or Δ33-85 mutant induces comparable levels of the IκB-ζ target gene IL-6 in the supernatant of the IκB-ζ-deficient U2932 cell line measured by ELISA. Error bars indicate the standard deviation. Western blotting indicates similar expression levels of IκB-ζ wildtype and Δ33-85 mutant.

** = p<0.01, *** = p<0.001

Figure S3: IκB-ζ expression is induced by MYD88 signaling.

shRNA-mediated knockdown of MYD88 downregulates IκB-ζ expression in HBL-1 and OCI-Ly3 cells measured by quantitative PCR and Western blotting.

** = p<0.01

Figure S4: IκB-ζ downregulation deregulates NF-κB activity in ABC DLBCL (see Figure 3).

A. Gene expression profiling following treatment with the IKKβ inhibitor MLN120b in HBL-1 cells. Changes of gene expression were profiled at the indicated timepoints and are depicted according to the color scale shown. Known NF-κB target genes and genes involved in processes such as inflammation are highlighted.

B. Gene expression profiling following treatment with the MALT1 inhibitor z-VRPR-fmk in HBL-1 cells. Changes of gene expression were profiled at the indicated timepoints and are depicted according to the color scale shown. Known NF-κB target genes and genes involved in processes such as inflammation are highlighted.
C. Gene set enrichment analysis of the z-VRPR-fmk defined gene expression signature. The signature is significantly downregulated following IκB-ζ knockdown.

D. Gene set enrichment analysis of a previously defined ABC DLBCL target gene expression signature (Supplemental Table 4). The ABC DLBCL target gene signature is significantly downregulated following IκB-ζ knockdown suggesting that the IκB-ζ gene signature is expressed at higher levels in primary ABC DLBCL patient samples compared to other malignant lymphoma subtypes.

E. NF-κB subunits p50 and p52 are expressed in the nucleus in ABC DLBCL cell lines (HBL-1, TMD8, OCI-Ly3, OCI-Ly10, and U2932). In contrast, the GCB DLBCL cell line BJAB does not harbor significant amounts of nuclear p50 and p52 respectively.
References


Nogai et al. Figure S2

A

Δ33-85 TMD8

Wildtype HBL-1

B

293T

NF-κB reporter (Firefly luciferase/Renilla luciferase)

Empty vector

κB-ζ wildtype

κB-ζ Δ33-85

C

U2932

Empty vector

κB-ζ wildtype

κB-ζ Δ33-85

Relative IL-6 expression (% of empty vector)

Empty vector

κB-ζ wildtype

κB-ζ Δ33-85
Nogai et al. Figure S3

[Graph showing relative IkappaB-z mRNA expression (% of control shRNA) for HBL-1 and OCI-Ly3 cells under control and MYD88 shRNA conditions.]

HBL-1

OCI-Ly3

Control shRNA MYD88 shRNA

Control shRNA MYD88 shRNA

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[Western blot images showing MYD88, IkappaB-z, and Tubulin for HBL-1 and OCI-Ly3 cells under control and MYD88 shRNA conditions.]
Nogai et al. Figure S4

A

IKK\(\beta\) inhibition:

Relative gene expression

2x

1x

0.5x

IKK\(\beta\) inhibition:

6h 12h 24h

B

MALT1 inhibition:

Relative gene expression

4x

1x

0.25x

MALT1 inhibition:

6h 12h 24h 48h

C

MALT1 target gene signature
(z-VRPR-fmk signature)

Enrichment score

Signature genes

Genes sorting metrics

p=0.001

D

ABC target gene signature

Enrichment score

Signature genes

Genes sorting metrics

p=0.001

E

Cytoplasmic extracts

Nuclear extracts

HBl-1 TMD8 OCI-Ly3 OCI-Ly10 U2932 BJAB HBl-1 TMD8 OCI-Ly3 OCI-Ly10 U2932 BJAB

p105

p50

p100

p52