SUPPLEMENTARY APPENDIX

Tumor necrosis factor receptor signaling is a driver of chronic lymphocytic leukemia that can be therapeutically targeted by the flavonoid wogonin

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Supplementary Methods

Samples and cell culture

All patients matched common standard diagnosis criteria for CLL. Isolation of PB mononuclear cells (PBMC), enrichment of $CD19^+$ B cells or $CD14^+$ monocytes, and set-up of cocultures were performed as described before.(1)

Statistical Analysis

Significance analyses for group comparisons were performed by unpaired or paired Student's *t* test or unpaired *t* test with Welch's correction using GraphPad software as indicated in the figure legends. Pairwise correlations were computed with Pearson's R. The effects of sTNFR-1 levels on survival endpoints were analyzed using log2 serum concentrations of sTNFR-1 as continuous variables in univariate Cox proportional hazard models and also in multivariate Cox models to assess their effects independently of clinical and laboratory parameters which were acquired as described before.(2) Hazard ratios were computed for a change of one unit on the log2 scale, i.e. for a two-fold change on the original scale. The association of overall survival (OS) and tumor-associated deaths (TAD) with log2 sTNFR-1 serum concentration levels as a continuous predictor was plotted by means of Stone-Beran plots using symmetrical nearest neighborhoods around the lowest, the median, and the highest observed values.(3-5) Statistical analyses of the effects of sTNFR-1 on survival endpoints were performed with the R statistical software, version 3.1 (R Core Team, 2014) and add-on packages survival, version 2.37, and prodlim, version 1.4.3.

Gene Expression Analysis

Microarray-based transcriptome analysis was performed using Human Oligo Set 4.0 chips (Operon, Cologne, Germany) as described before.(1) The data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE100801.

Transcript levels were further analysed by quantitative reverse transcription-PCR using the ΔΔCt method relative to three internal controls (*HPRT, DCTN2, PGK*) as described.(1)

Flow cytometry

Cell survival was assessed after annexin V-PE and 7-amino-actinomycin (7-AAD) staining as described before.(6) Cell proliferation was monitored using Click-It EdU (5-ethynyl-2´-deoxyuridine) flow cytometry assay, and Alexa Fluor 488 azide (Invitrogen, USA) as described.(7) Quantification of mTNFR-1 on CLL cells was performed on CD20-positive cells by using the following antibodies and respective isotype controls after pre-blocking with 2% human immunoglobulin cocktail (Sigma-Aldrich): anti-CD20 (clone: L27), biotin-anti-TNFR-1 (clone: MABTNFR1-B1), PE-streptavidin conjugate (BD Pharmingen, Germany). Single cell suspensions of murine tissues were prepared and stained with the following antibodies after blocking with 4% rat serum (Stemcell Technologies, Germany): anti-CD5 (clone: 53-7.3), anti-CD19 (clone: eBio1D3), anti-CD45 (clone: 30F-11), and anti-TNFR-1 (clone: 55R-170), and respective isotype control antibodies. Relative median fluorescence intensity was calculated as ratio of values obtained by specific versus control antibody staining. All measurements were carried out on a FACS CantoII or LSRFortessa flow cytometer and analyzed using FACSDiva software (BD Biosciences).

Tissue Microarrays and Immunohistochemistry

Primary antibody reaction was detected using a polymer-based peroxidase-labeled system (Super Sensitive Polymer-HRP, BioGenex) with VIP used as the chromogen. Due to differences in antigen retrieval requirements between TNFR-1 and CD20, CD3 and CD68, the TNFR-1 was stained first and sections were scanned using a digital pathology system (Pannoramic 250 Flash, 3DHistec). Sections were then stripped using a heat-induced epitope retrieval method and subsequently re-stained using either anti-CD20, -CD3 or -CD68 and then re-scanned on the same system. Using the slide linking feature, each tissue core was reviewed manually to determine co-staining of antibodies. Evaluation of percentage TNFR-1 positive cells on stained slides was performed using a computerized image

analysis system (Ariol, Leica Microsystems) on the basis of pathologist-trained visual parameters for colour and shape. Each core was assessed and acellular and fibrotic portions were excluded prior to analysis and only tumor-rich areas were included. Analysis areas were restricted to follicles in the normal and reactive LN for a valid comparison. Only hematopoietic areas were included in the normal BM trephines.

NFκB Activity

Activation of the canonical NFκB pathway was assessed via an oligonucleotide-based chemiluminescence ELISA using a p65-specific antibody as described before.(8)

Animal work

All animal work was carried out under UK Home Office Project Licence PPL 70/7531 “Characterisation & repair of immune defects in CLL”, in accordance to the Animals Act 1986.

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Supplementary Table S1: Clinical variables of serum samples from 247 patients of the German CLL8 study cohort that were used for quantification of sTNFR-1. All Patients were Binet stage B or C and required clinical intervention.

Clinical variable	Levels	n	%
IGHV	M	89	36
	UM	147	59.5
	n.a.	11	4.5
***TP53* mutation**	no	207	83.8
	yes	29	11.7
	n.a.	11	4.5
***NOTCH1* mutation**	no	149	60.3
	yes	8	3.2
	n.a.	90	36.4
***SF3B1* mutation**	no	129	52.2
	yes	28	11.3
	n.a.	90	36.4
Del(11q)	no	169	68.4
	yes	63	25.5
	n.a.	15	6.1
Del(17p)	no	215	87.0
	yes	17	6.9
	n.a.	15	6.1
Del(13q)	no	106	42.9
	yes	125	50.6
	n.a.	16	6.5
Del(6q)	no	42	17.0
	yes	0	0.0
	n.a.	205	83.0
Tris12	no	201	81.4
	yes	30	12.2
	n.a.	16	6.5
Genetic hierarchy	Del(13q)	34	13.8
	normal	23	9.3
	Tris12	13	5.3
	Del(11q)	34	13.8
	Del(17p)	9	3.6
	other	1	0.4
	n.a.	133	53.9

M = mutated *IGHV* genes
UM = unmutated *IGHV* genes
n.a. = not assessed

Supplementary Table S2: Multivariate analysis of overall survival and tumor-associated deaths**.**

	Overall survival			Tumor-associated deaths		
	HR[a]	**95% CI[b]**	**P value**	**HR[a]**	**95% CI[b]**	**P value**
Univariate analysis						
log2(sTNFR-1)	1.381	1.018 - 1.873	0.038	1.3456	0.9831 - 1.8418	0.064
Multivariate analysis						
log2(sTNFR-1)	1.5997	1.1525 - 2.2204	0.005	1.5800	1.1284 - 2.2123	0.008
Rituximab treatment	0.8437	0.5656 - 1.2585	0.405	0.8352	0.5529 - 1.2618	0.392
Age (10 years)	1.3185	1.0122 - 1.7175	0.040	1.3233	1.0075 - 1.7382	0.044
11q deletion	0.8173	0.5117 - 1.3055	0.399	0.8623	0.5319 - 1.3978	0.548
17p deletion	7.1045	3.0941 - 16.3132	< 0.001	7.6194	3.2876 - 17.6585	< 0.001
IGHV mutational status	2.3692	1.4486 - 3.8749	0.001	2.1602	1.3181 - 3.5405	0.002

[a] Hazard ratio (HR) estimated from Cox proportional regression model.
[b] Confidence interval (CI) of the estimated HR.

Supplementary Table S3: Differentially expressed genes obtained by significance analysis of microarray data (SAM) showing mean log_2 expression changes on day 1 after high cell density culture compared to day 0 in CLL cells (n = 4) and healthy donor (HD) B cells (n = 5). Results are sorted according to the difference between CLL and HD expression changes.

GeneSymbols	Mean CLL	Mean HD	Difference CLL - HD
ARRDC4	0,88	-2,17	3,05
MYADM	-1,05	-3,59	2,54
TNFRSF1A	**2,06**	**-0,18**	**2,24**
FTHL16;RP4-646B12.2;AC104820.1;AC023644.6;RAB7A	0,18	-1,97	2,15
CCDC93;AC009404.5	-0,78	-2,89	2,11
FTHL16;RSF1;RP11-518K17.3;AL354828.12-2;AC104820.1;RP11-274P12.1;AP000609.2;AC026785.4-2	0,12	-1,95	2,07
LOH12CR1	0,08	-1,81	1,88
RGPD2;RGPD1;RGPD3;PLG;PLGLB1;PLGLB2;PLGLA1	0,33	-1,30	1,64
PHF20	0,20	-1,40	1,60
RCOR1	-0,17	-1,74	1,57
FAM49A	-0,45	-1,89	1,44
SH3GL1	-0,04	-1,30	1,26
JAM3	-0,05	-1,26	1,21
RDH10	0,89	-0,27	1,16
SIPA1	0,79	-0,30	1,10
CHD2	-0,68	-1,77	1,08
NPIPL3;SMG1;AC008740.7-2;AC106782.5-2;AC009060.7-2;AC130466.2;AC025279.6-2	-0,12	-1,12	1,00
LGALS8;RP11-385F5.4	0,78	-0,21	0,99
LGALS8;RP11-385F5.4	0,54	-0,42	0,96
INF2	0,47	-0,48	0,96
DAP	0,34	-0,58	0,92
BRI3;DBT;RP11-305E17.5	-0,05	-0,84	0,79
ALAS1	1,34	0,57	0,77
GNMT	0,25	-0,51	0,75
KIAA1219	0,00	-0,74	0,74
SMURF2	-0,38	-1,06	0,68
BCKDHB	-0,61	1,32	-1,93
ATG4C	0,59	2,52	-1,93
DUSP4	-1,52	0,47	-1,99

MINA	0,20	2,19	-2,00
PRKAR1B	-0,12	1,90	-2,02
JAZF1	0,09	2,11	-2,02
ALDH2	0,61	2,64	-2,03
MAPKAP1	-0,55	1,48	-2,04
C20orf30;AP004245.2	0,51	2,63	-2,12
KIAA0146	1,18	3,31	-2,13
FCRL3	0,39	2,53	-2,14
CYB5R2	-0,37	1,85	-2,22
ODC1	-2,74	-0,51	-2,23
RTN4IP1	-0,17	2,06	-2,23
KLHL14	-0,45	1,80	-2,25
HDDC2	0,07	2,35	-2,29
FXYD2;AP000757.4	0,79	3,09	-2,30
IMMP2L	-0,39	1,97	-2,36
ARHGAP24	-2,18	0,19	-2,38
SLC38A5	0,65	3,08	-2,42
C10orf104	0,09	2,57	-2,49
KLHL14	-0,47	2,02	-2,49
FAM173B	1,23	3,75	-2,52
CTSH	-0,30	2,24	-2,54
IGLV3-1	-1,56	1,10	-2,67
GPT2	-0,22	2,71	-2,93
FCRLA	0,70	3,91	-3,22
ENTPD1;RP11-429G19.2	-0,03	3,40	-3,43
NME1-NME2	-0,17	3,27	-3,44
PSAT1;RP11-548C21.1	1,51	5,17	-3,66
CD1C	-1,37	2,35	-3,73
FABP5;KB-67B5.16;AKAP13;ATP7B;STX3	-0,75	2,99	-3,73

Supplementary Table S4: Patients' data, including clinical stage at time of investigation, fluorescence in situ hybridization (FISH) results, mutational status of *IGHV* genes, ZAP-70 results, and assays performed.

Patient ID	Clinical stage (Binet)	FISH result	*IGHV* mutational status	ZAP70	Assays performed
CLL 283	A	Del(13q)	UM	n.a.	mTNFR-1
CLL 305	C	Tris12	UM	n.a.	mTNFR-1
CLL 237	B	Del(13q)	UM	n.a.	mTNFR-1
CLL 226	n.a.	Del(17p) Del(13q)	UM	pos.	mTNFR-1
CLL 187	A	Del(13q)	UM	neg.	mTNFR-1
CLL 303	B	Del(11q) Del(13q)	M	n.a.	mTNFR-1
CLL 648	B	Del(11q) Del(13q)	UM	neg.	mTNFR-1
CLL 173	A	Del(13q)	M	n.a.	mTNFR-1
CLL 297	A	Del(13q)	M	neg.	mTNFR-1
CLL 298	C	Del(13q)	M	neg.	mTNFR-1
CLL 208	C	Del(13q)	M	neg.	mTNFR-1
CLL 229	B	Del(13q)	M	n.a.	mTNFR-1
CLL 398	n.a.	Del(13q)	M	neg.	mTNFR-1
CLL 383	A	normal	M	pos.	mTNFR-1
CLL 384	B	normal	M	n.a.	mTNFR-1
CLL 385	A	Del(13q)	M	neg.	mTNFR-1
CLL 316	A	Del(13q)	M	n.a.	mTNFR-1
CLL 317	C	Del(13q)	M	n.a.	mTNFR-1
CLL 319	C	normal	M	neg.	mTNFR-1
CLL 181	B/C	Del(13q)	n.a.	n.a.	mTNFR-1
CLL 298	C	Del(13q)	M	neg.	mTNFR-1
CLL 308	C	Del(13q)	n.a.	n.a.	mTNFR-1
CLL 178	n.a.	n.a.	n.a.	n.a.	mTNFR-1

CLL 201	n.a.	n.a.	n.a.	n.a.	mTNFR-1
CLL 93	n.a.	Del(13q)	M	neg.	qRT-PCR *TNFR1*
CLL 98	n.a.	n.a.	UM	n.a.	qRT-PCR *TNFR1*
CLL 99	n.a.	Del(13q)	M	neg.	qRT-PCR *TNFR1*
CLL 100	n.a.	13q- bidel	M	n.a.	qRT-PCR *TNFR1*
CLL 208	n.a.	Del(13q)	M	neg.	wogonin
CLL 229	B	Del(13q)	M	n.a.	wogonin
CLL 242	C	Del(13q)	M	neg.	wogonin
CLL 173	A	Del(13q)	M	n.a.	wogonin
CLL 318	A	Del(13q)	UM	n.a.	wogonin
CLL 333	A	normal	M	pos.	NFκB TNF/wogonin
CLL 334	C	Del(13q)	M	neg.	NFκB TNF/wogonin
CLL 442	n.a.	n.a.	n.a.	n.a.	NFκB TNF-α
CLL 409	n.a.	n.a.	n.a.	n.a.	NFκB TNF-α
CLL 423	n.a.	n.a.	n.a.	n.a.	NFκB TNF-α

FISH = fluorescence in situ hybridization
M = mutated *IGHV* genes
UM = unmutated *IGHV* genes
n.a. = not assessed
neg. = negative
pos. = positive
A, B, C = Binet stage A, B, C