Supplementary Material

BAFF/APRIL System is Functional in B-Cell Acute Lymphoblastic Leukemia in a Disease Subtype Manner

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Supplementary Methods

mRNA analysis

To detect the transcript levels of *BAFFR* and *TACI*, cDNA was amplified by specific primers in duplicate quantitative Real-time RT-PCR reactions using Platinum-SYBR-Green qPCR Supermix (Invitrogen, Waltham, Massachusetts, USA) in the Rotor-Gene 6000 cycler [Corbett Life Science, Asia-Pacific (APAC), Australasia], and all measurements were averaged, as described elsewhere (1). The *B2M* gene was used as a reference gene, correcting for variations in mRNA recovery and reverse transcription yield. Relative quantification and calculation of the range of confidence were performed using the comparative $\Delta\Delta^{CT}$ method, as described by Livak (2) and the relative expression of each gene is presented as a multiple of the respective gene expression in isolated B-cells of a healthy individual.

BCMA, *APRIL*, *BAFF* and *ΔBAFF* mRNA levels were detected by amplifying cDNA products in the presence of 1μM of specific primer in a semi-quantitative conventional RT-PCR using MyTaq DNA polymerase (Bioline, London, UK) in an Applied Biosystems Verity Thermal Cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). PCR products were separated on a 2% agarose gel containing ethidium bromide and visualised by the Uvitec gel documentation and analysis system (UVItec Limited, Cambridge, UK) with the aid of the GelCapture acquisition software (DNR Bio-Imaging systems, Neve Yamin, Israel). The quantification of the PCR fragments was performed by the UVIdoc software (UVItec Limited). The expression levels of *BCMA*, *APRIL*, *BAFF* and *ΔBAFF* were calculated as the intensity quotient for each gene to *B2M*.

The primers for the amplification of *TACI*, *BAFF* and *ΔBAFF* were described previously (1, 3), while the primers for the amplification of *BAFFR*, *BCMA*, and *APRIL* were designed with the aid of the Oligo 6.0 software (NBI, Plymouth, MN, USA). The semi-quantitative conventional RT-PCR conditions for each gene were set up so that none of the analyzed RNAs reached a plateau, meaning that they were at the middle of the exponential phase of amplification.

Concerning the detection of TEL-AML1, E2A-PBX1, BCR-ABL (p190 ALL- and p210 CML-type) translocations in patients with ALL, we performed a multiplex two-step RT-PCR as described by Arrifin *et al.* (4). The cell lines K562 (positive for BCR-ABL/p210 rearrangement) and 697 (positive for E2A-PBX1 rearrangement), as well as patients' samples with previously established rearrangements for BCR-ABL/p190 and TEL-AML1 were used as positive controls in our experiments. For the confirmation of results, all PCR products with suspected rearrangements were purified by QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and directly sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a Big Dye Terminator DNA sequencing kit (Applied Biosystems).

Cell isolation

For B-cell survival assays, B-ALL lymphoblasts from patients and normal peripheral blood B-cells were enriched from mononuclear cells by magnetically depleting all non-B-cells. In particular, cells were incubated with FITC-labelled antibodies against CD2, CD3, CD14, CD16, CD36, CD43 (Beckman Coulter, Brea,

CA, USA) with the exception of patients with B-ALL, where the anti-CD43 antibody was not included in the staining, as malignant B-lymphoblasts express the molecule. Afterwards, all non-B-cell subsets were positively isolated with the aid of the EasySep™ Human FITC Positive Selection Kit (Stemcell Technologies, Vancouver, USA). Purity of the isolated B-cells (malignant or not) was assessed by flow cytometry with a PE-labelled anti-CD19 antibody (Beckman Coulter).

For the measurement of mRNA levels of BAFF/APRIL ligands and receptors, B-cells and monocytes were also isolated from PBMCs of healthy donors. B-cells were isolated after labelling with a PE-conjugated anti-CD19 antibody (Beckman Coulter), followed by positive selection with EasySep™ Human PE Positive Selection Kit (Stemcell Technologies). For monocyte isolation, cells were negatively selected using the FITC-conjugated antibodies CD2, CD4, CD8, CD19, CD43 (Beckman Coulter) and the EasySep™ Human FITC Positive Selection Kit that was mentioned above.

Supplementary Tables

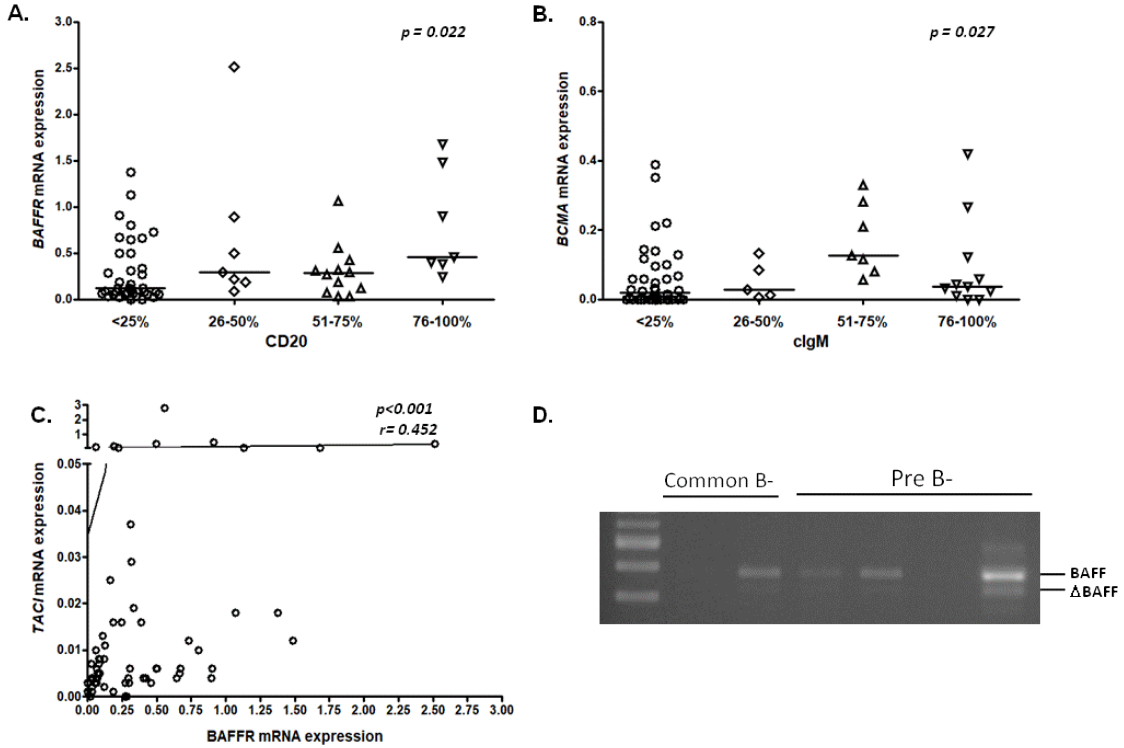
Supplementary Table 1. Primers used in this study.

Gene	Forward primer(5' →3')	Reverse primer(5' →3')
Quantitative Real Time RT-PCR		
BAFFR	GAGACAAGGACGCCCCAGA	GGTCACCAGTTCAGTGGAGC
TACI	TCTGCCTGTGTGCCGTCCTC	CGGCTTCCATCGCGTGAT
B2M	Commercially obtained by SAbiosciences (cat no. PPH01094E)	
Semi-quantitative conventional RT-PCR		
BCMA	TTGGGACTGAGCTTAATAAT	CGGAAGAATAATTTCATCAC
BAFF & ΔBAFF	GAGTCTCCCGGAGCAGAGTT	GCAGGAATTATTGGGTAGTGT
APRIL	GAGTCTCCCGGAGCAGAGTT	CTTGGAGGTGGCGTTAATGG
B2M	CTATCCAGCGTACTCCAAAG	GAAAGACCAGTCCTTGCTGA
Real Time PCR for ChIP		
BAFFR promoter	GGCGCTATCTCGGCTCA	GCGCCTACAATCTCAGCTAC
BAFFR 2nd intron	GGGCAGGGAGTTGGGG	TTGGGAGTCTAACGGAGCTT
BAFFR 3'UTR	AAATGGCATCTTAGGTTCTG	GCTTGCTTTGGGATTTAGT
Negative control region	CCCATGAAGACCCAGGCAAG	GGCGAGTCCAGAGCAGTGTG

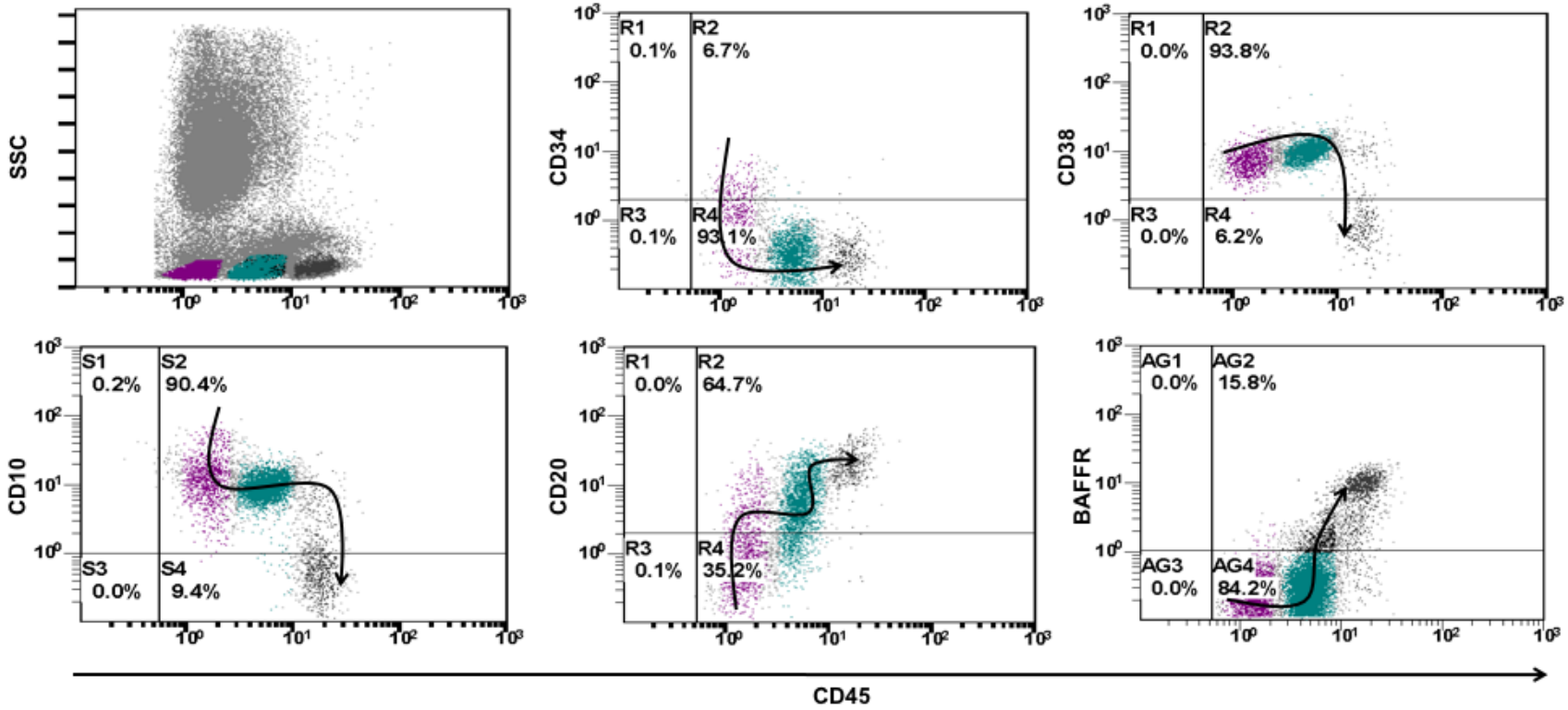
Supplementary Table 2. BAFF promotes cell death of B-lymphoblasts from adult patients with BCR-ABL$^+$ pre-B-ALL. Isolated blasts from 5 patients with B-ALL were cultured for 5 and 17 hours with 60-mer BAFF (0.049-12.5 ng/mL). Normal peripheral CD19$^+$ cells were evaluated after 3 days, serving as positive controls; each condition was tested in triplicates. The number of CD19$^+$ 7AAA-D$^-$ cells was determined by flow cytometry (mean ± standard deviation); patient 64: BAFFR$^-$ common B-ALL, patient 71: BAFFR$^+$ common B-ALL, patients 65 & 69: BAFFR$^+$ BCR-ABL$^+$ pre-B-ALL, patient 72: BAFFR$^-$ pro-B-ALL, controls 1-3: CD19$^+$ cells isolated from peripheral blood mononuclearcells of 3 healthy donors.

		BAFF (ng/mL)				
Patient no.	**Control**	**0.049**	**0.195**	**0.781**	**3.125**	**12.5**
Patient 64 (5h)	11100 ± 315	11686 ± 742	11343 ± 356	11025 ± 57	11093 ± 172	11135 ± 127
Patient 64 (17h)	10044 ± 51	10523 ± 287	10495 ± 224	10551 ± 88	10106 ± 152	10116 ± 174
Patient 71 (5h)	10768 ± 564	10254 ± 373	10627 ± 408	11012 ± 523	10489 ± 83	10458 ± 107
Patient 71 (17h)	7471 ± 129	7394 ± 135	7564 ± 64	7626 ± 353	7766 ± 325	7569 ± 405
Patient 65 (5h)	2354 ± 24	1697 ± 32	1791 ± 134	1937 ± 104	1964 ± 93	2014 ± 43
Patient 65 (17h)	478 ± 35	361 ± 11	352 ± 20	420 ± 18	391 ± 52	448 ± 38
Patient 69 (5h) – 1st experiment	6412 ± 591	5692 ± 114	5358 ± 220	5527 ± 142	5575 + 69	5522 ± 114
Patient 69 (17h)– 1stexperiment	2295 ± 65	1281 ± 32	982 ± 90	881 ± 124	1278 ± 417	888 ± 185
Patient 69 (5h)– 2nd experiment	6346 ± 221	5766 ± 64	5544 ± 230	5668 ± 289	5901 ± 91	6138 ± 117
Patient 69 (17h)– 2nd experiment	2453 ± 40	1269 ± 265	1221 ± 60	1257 ± 40	1469 ± 51	1685 ± 90
Patient 72 (5h)– 1stexperiment	11939 ± 71	12450 ± 346	11740 ± 331	11855 ±303	11779 ± 196	11529 ± 91
Patient 72 (17h)– 1stexperiment	7709 ± 508	7251 ± 118	7147 ± 144	6990 ± 102	7215 ± 223	7078 ± 140
Patient 72 (5h)– 2nd experiment	11474 ± 123	11189 ± 229	11366 ± 777	11129 ± 99	11449 ± 153	11868 ± 286
Patient 72 (17h)– 2nd experiment	8251 ± 420	7563 ± 98	7281 ± 150	7310 ± 324	7566 ± 126	7777 ± 120
Patient 69 (5h) after induction therapy	6155 ± 185	5817 ± 35	5799 ± 256	5830 ± 105	5823 ± 140	6170 ± 232
Patient 69 (17h) after induction therapy	2433 ± 165	1616 ± 114	1580 ± 190	1685 ± 186	1875 ± 164	2087 ± 137
Control #1	1797 ± 133	2407 ± 155	3378 ± 98	3855 ± 149	3442 ± 405	3216 ± 191
Control #2	5986 ± 762	6628 ± 156	7620 ± 112	8415 ± 324	8013 ± 186	7172 ± 149
Control #3	3390 ± 147	3900 ± 144	4611 ± 70	5107 ± 72	4750 ± 220	4466 ± 107

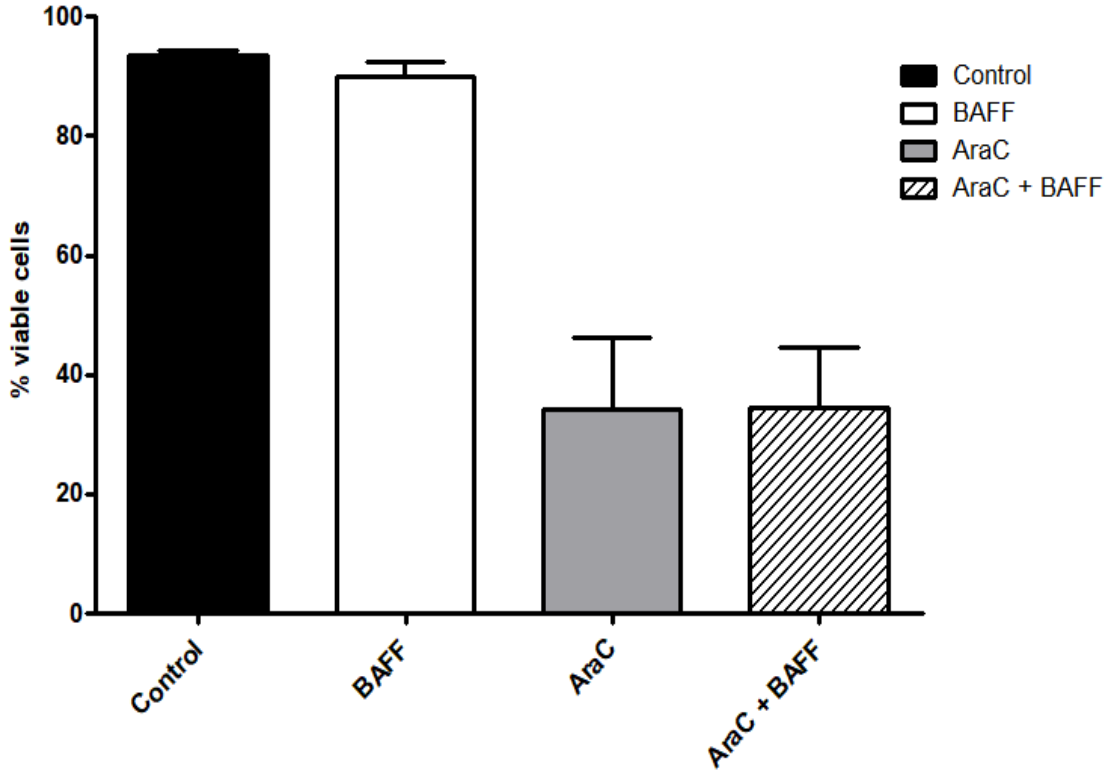
Supplementary Figures



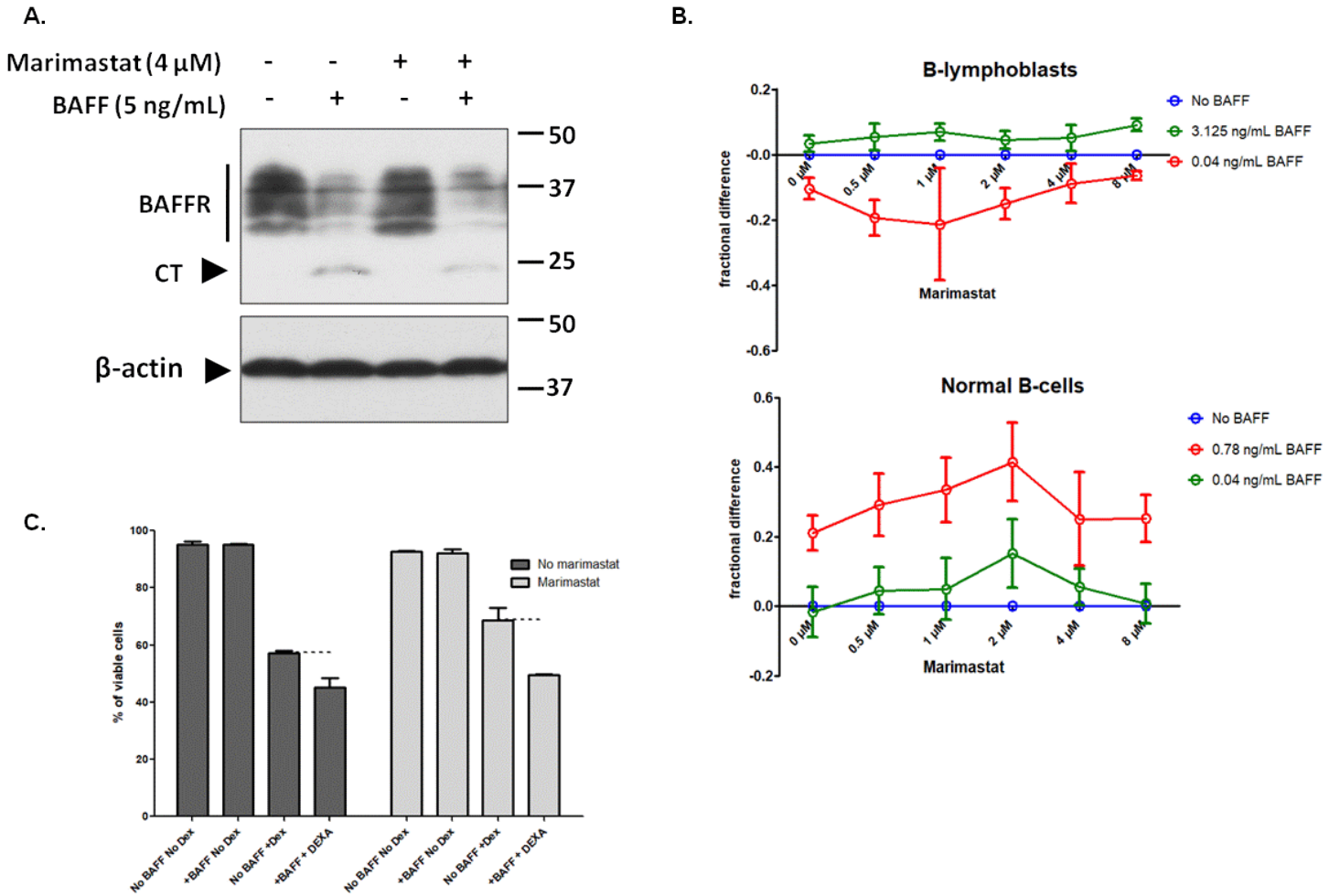
Supplementary Figure 1. Correlation of receptors expression at mRNA level. (A) *BAFFR* mRNA levels are correlated with the surface expression (%) of CD20. **(B)** *BCMA* mRNA levels are correlated with the expression (%) of cytoplasmic IgM; *p-value* in each plot refers to Kruskal-Wallis *H* test. **(C)** *TACI* mRNA levels are positively associated with *BAFFR* mRNA levels; p-value refers to Spearman's rank correlation coefficient. **(D)** Representative data of mRNA expression of *BAFF* and *ΔBAFF* in patients with common and pre-B-ALL.



Supplementary Figure 2. Representative expression pattern of antigens during B-cell maturation. Dot plots are gated in $CD19^{+}$ cells. The arrows show the direction of maturation pathway. BAFFR is not expressed in normal early stage B-cell progenitors.
Violet cells: CD34+ CD45low, CD38hi, CD10hi CD20lo/- BAFFR- (CLP and pro-B cells)
Green cells: CD34- CD45int, CD38hi, CD10hi, CD20 int, BAFFR- (pre-B and immature B cells)
Black cells: CD34- CD45+/high, CD38low, CD10-, CD20+ BAFFR+ (recirculating mature B cells)



Supplementary Figure 3. BAFF does not affect the aracytine-mediated apoptosis of 697 cells. Percentage of viable cell counts (Annexin V^- 7-AAD^- cells) of 697 cells incubated with/without 3-mer BAFF and with/without 160 ng/mL aracytine (AraC) for 5 days.



Supplementary Figure 4. Marimastat does not alter the BAFF-mediated spontaneous or dexamethasone-induced apoptosis of primary B-lymphoblasts and 697 cells, respectively. (A) 697 cells were incubated overnight with/without 4 µM Marimastat and with/without 5 ng/mL 60-mer BAFF. Cell lysates were blotted using an antibody that detects the C-terminal (CT) fragment of BAFFR. **(B)** Primary B-lymphoblasts of

a BAFFR^{+} BCR-ABL^{+} pre-B-ALL patient and pure normal B-cells were treated with increasing concentrations of marimastat and 2 concentrations of 60-mer BAFF (one optimal and one suboptimal according to previous survival assays). At the indicated time points (5h & 17h for B-lymphoblasts, 3 days for normal B-cells), the number of CD19^{+} 7-AAD^{-} cells was evaluated. Plots show the fractional difference [calculated as (cell number in the presence of BAFF - cell number w/o BAFF) / cell number w/o BAFF, for each concentration of marimastat respectively] between treatment conditions in B-lymphoblasts (17h) and in pure normal B-cells. **(C)** 697 cells were incubated for 3 days with/without 8 μM marimastat, in the presence or not of 60-mer BAFF (5 ng/mL) and/or 20 ng/mL dexamethasone (dex). Despite the fact that marimastat opposes to dexamethasone action, 697 cells retain their ability to display increased cell death in response to BAFF and dexamethasone treatment, regardless of the presence of the pan-metalloprotease inhibitor. Plots show mean of percentage of viable cell counts (Annexin V^{-} 7-AAD^{-} cells) of 697 cells.

References

1. Mamara A, Germenis AE, Kompoti M, Palassopoulou M, Mandala E, Banti A, et al. TACI expression and signaling in chronic lymphocytic leukemia. J Immunol Res (2015) 2015:478753.

2. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (2001) 25:402-8.

3. Novak AJ, Slager SL, Fredericksen ZS, Wang AH, Manske MM, Ziesmer S, et al. Genetic variation in B-cell-activating factor is associated with an increased risk of developing B-cell non-Hodgkin lymphoma. Cancer Res (2009) 69:4217-24.

4. Ariffin H, Chen SP, Wong HL, Yeoh A. Validation of a multiplex RT-PCR assay for screening significant oncogene fusion transcripts in children with acute lymphoblastic leukaemia. Singapore Med J (2003) 44:517-20.