Microenvironmental stromal cells abrogate NF-κB inhibitor-induced apoptosis in chronic lymphocytic leukemia

Carl Philipp Simon-Gabriel,[1*] Katharina Foerster,[1*] Shifa Saleem,[1] Dorothee Bleckmann,[1] Marco Benkisser-Petersen,[1] Nicolas Thornton,[1] Kazuo Umezawa,[2] Sarah Decker,[1] Meike Burger,[3] Hendrik Veelken,[4] Rainer Claus,[1] Christine Dierks,[1] Justus Duyster[1] and Katja Zirlik[1,5]

[1]Department of Hematology, Oncology and Stem Cell Transplantation, University Medical Center, Faculty of Medicine, University of Freiburg, Germany; [2]Department of Molecular Target Medicine, Aichi Medical University School of Medicine, Nagakute, Japan; [3]Furtwangen University, Faculty of Medical and Life Sciences, Schwenningen Campus, Villingen-Schwenningen, Germany; [4]Department of Hematology, Leiden University Medical Centre, the Netherlands and [5]Tumor and Breast Center ZeTuP, St. Gallen, Switzerland

**CS-G and KF contributed equally to this work*

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Correspondence: katja.zirlik@zetaup.ch / katja.zirlik@uniklinik-freiburg.de

Supplementary methods

Reagents.

As culture medium, RPMI (RPMI 1640 Medium (1x) + L-Glutamin, Gibco) containing 10% fetal calf serum (FCS, Sigma) and 1% penicillin/streptomycin (Gibco) was used.

DHMEQ is a NF-κB inhibitor blocking nuclear translocation and DNA binding of NF-κB and was prepared in Aichi Medical University. Fludarabine was purchased from Sigma-Aldrich. Idelalisib, Ibrutinib, and R406 were obtained from Selleckchem. All inhibitors were dissolved in DMSO, which was also used as a negative control. Recombinant human BAFF (rhBAFF), rhAPRIL, soluble CD40 ligand (sCD40L), and CXCL12 (also known as SDF-1α) were purchased from PeproTech.

Subcellular fractionation and detection of cytoplasmatic and nuclear NF-κB.

Cells were collected by centrifugation and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in 500 µl hypotonic buffer (20mM Tris-HCl (pH 7.4), 10mM NaCl, 3mM $MgCl_2$) and incubated for 15min. 25 µl NP4O (10%) were added, the lysate was spun and the supernatant was collected as the cytoplasmic fraction. The remaining pellet was resuspended in 50µl cell extraction buffer (10mM TRIS (pH 7.4), 2mM Na_3VO_4, 100mM NaCl, 1% Triton-X-100, 1mM EDTA, 10% Glycerol, 1mM EGTA, 0,1% SDS, 1mM NaF, 0,5% deoxycholate, 20mM $Na_4P_2O_7$), incubated for 30min and spun for 30min. The supernatant was then recovered as nucleus lysates.

Western blot antibodies.

The NF-κB Family Member Antibody Sampler Kit, the rabbit monoclonal antibodies against ß-Actin, PARP, TRAF-1, Histon H3 XP and Mcl-1 were purchased from Cell Signaling Technology (Danvers, MA) and the rabbit monoclonal antibodies against Bax, Bcl-2 and Bcl-Xl were purchased from Abcam (Cambridge, UK).

Analysis of the effect of concomitant versus sequential combination of DHMEQ with Ibrutinib or R406 respectively.

CLL cells were co-cultured with M2-10B4 cells at a ratio of 20:1. For concomitant treatment DHMEQ (5µg/ml) + Ibrutinib (10µM) or DHMEQ (5µg/ml) + R406 (5µM) were added at the same time. For sequential treatment DHMEQ (5µg/ml) was added first and Ibrutinib (10µM) or R406 (10µM) were added 4 hours later and vice-versa (Ibrutinib or R406 first, DHMEQ 4 hours later). Cell viability of the harvested CLL cells was measured by flow cytometry using annexin V/propidium iodide staining after 48h of treatment.

Analysis of synergistic drug effects on CLL cells co-cultured with M2-10B4 cells.

CLL cells were co-cultured with M2-10B4 cells at a ratio of 20:1. For quantitative determination of potential synergistic drug interaction between DHMEQ and Ibrutinib as well as DHMEQ and R406, DHMEQ was titrated from 8µg/ml, Ibrutinib was titrated from 15µM and R406 was titrated from 10µM respectively. For combined treatments, DHMEQ and Ibrutinib were titrated at a constant ratio of 1:1.875, and DHMEQ and R406 at a constant ratio of 1:1.25. Viability was measured after 48h by flow cytometry using Annexin

V/Propidium iodide staining. Drug-induced cytotoxicities were calculated as follows: 1-(viability $_{(treated)}$/ viability($_{control)}$). By applying the median-effect principle and the multiple drug effect equation described by Chou and Talalay[1] using the CompuSyn software (ComboSyn Inc.), Combination Indices and Dose-Reduction Indices were calculated. A Combination Index <1, =1 and >1 indicates synergism, additive effect and antagonism, respectively. A Dose-reduction Index (DRI)>1 indicates favorable dose-reduction, while a DRI<1 indicates unfavorable dose reduction.

References.

1. Chou T-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. Pharmacol Rev 2006;58(3):621–681.

Supplementary figures

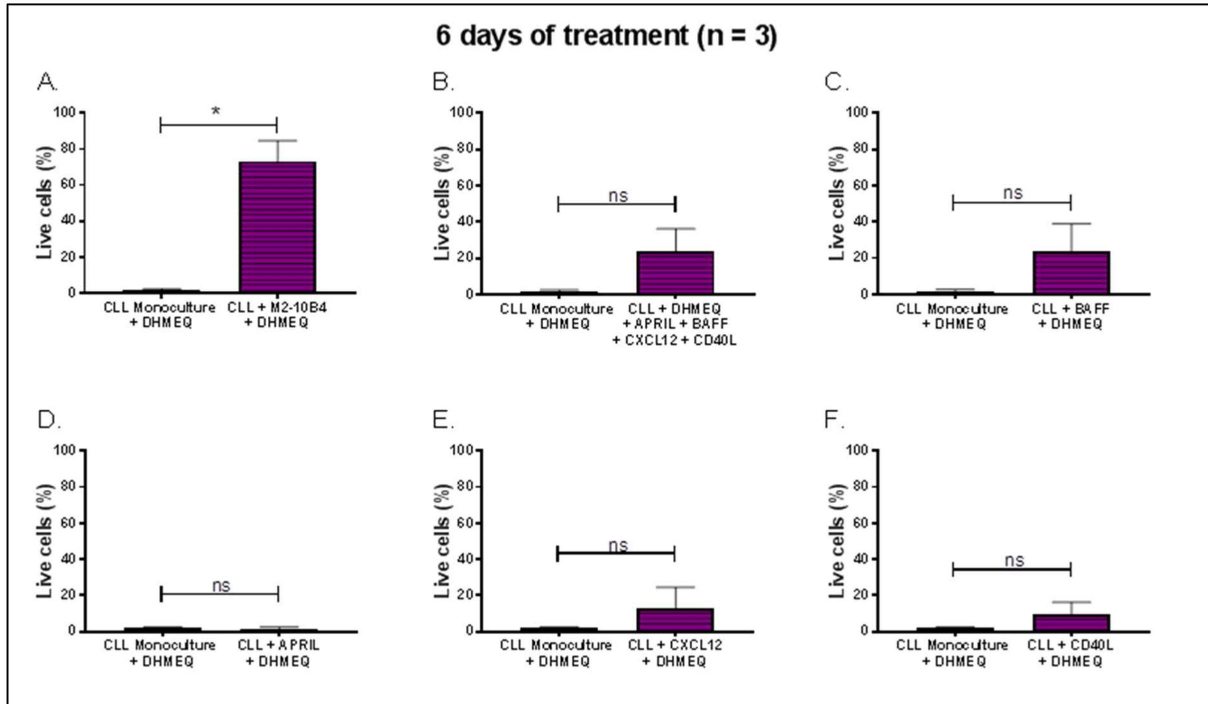

Fig. S1: BAFF protects CLL cells better from DHMEQ-induced apoptosis than APRIL, CD40L or CXCL12.

Cell viability as measured by flow cytometry after ANX5/PI staining of CLL cells treated with DHMEQ (2µg/ml) with or without (A) M2-10B4 cells, (B) APRIL (500µg/ml), BAFF (50ng/ml), CXCL12 (100ng/ml) and CD40L (1µg/ml), (C) BAFF only (50ng/ml), (D) APRIL only (500µg/ml), (E) CXCL12 only (100ng/ml) or (F) CD40L only (1µg/ml). Statistical analysis: Welch's t-test.

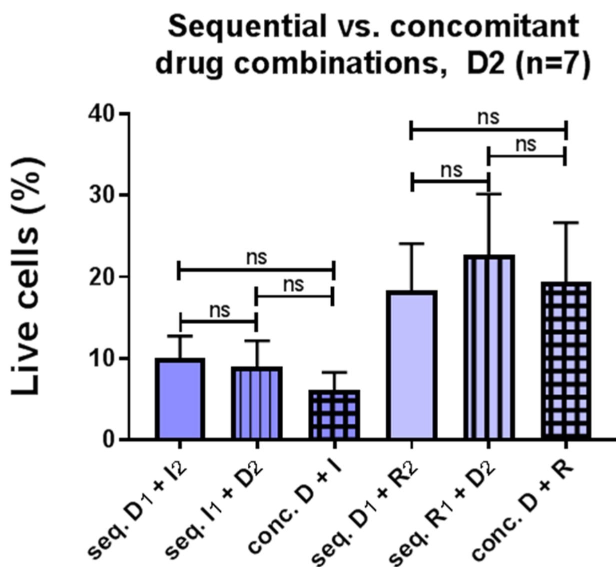

Figure S2 – No response improvement by sequential drug combinations vs. concomitant drug treatments.

Cell viability as measured by flow cytometry of M2-10B4 co-cultured CLL cells treated for 48h with DHMEQ (D; 5µg/ml) in combination with either Ibrutinib (I; 10µM) or R406 (R; 5µM), the drugs being added sequentially (time interval of 4 hours) or concomitantly (drugs added at the same time). From left to right: DHMEQ added 4h before Ibrutinib; Ibrutinib added 4h before DHMEQ; DHMEQ and Ibrutinib added together; DHMEQ added 4h before R406; R406 added 4h before DHMEQ; DHMEQ and R406 added together.