Mesenchymal stromal cells protect mantle cell lymphoma cells from spontaneous and drug-induced apoptosis through secretion of B-cell activating factor and activation of the canonical and non-canonical nuclear factor κB pathways

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Online Supplementary Design and Methods

Flow cytometric analysis for surface membrane expression of B-cell activating factor and its receptors

To analyze for cell surface expression of B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), or B-cell activating factor receptor (BAFFR), mantle cell lymphoma (MCL) cells ($1x10^5$/100 μL) were stained with saturating amounts of the appropriate phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibody (eBioscience, San Diego, CA, USA) for 30 min at 4°C in FACS buffer (phosphate-buffered saline supplemented with 0.5% bovine serum albumin). After having been washed twice in FACS buffer, the cells were analyzed using a FC500 flow cytometer (Beckman Coulter Immunology, Hialeah, FL, USA). In each experiment the appropriate PE- or FITC-conjugated isotype control antibody was used to determine background staining.

Western blotting

MCL cells were cultured alone or co-cultured with human mesenchymal stem/stromal cells (hMSC) or MS5 stromal cells. After 5 days in culture, cells were harvested, washed and lysed using lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, EDTA, 2 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethyl sulfonyl fluoride, 5 g/mL leupeptin, and 5 g/mL aprotinin). Cell lysates were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and immunoblotted. Specific antibodies for bcl-2, and PARP, were purchased from Cell Signaling Technology (Beverly, MA, USA). Antigen-antibody complexes were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). Blots were stripped and re-probed with anti-actin antibody (Sigma-Aldrich, St Louis, MO, USA) to ensure equivalent protein loading.

Annexin V-propidium iodide staining

Apoptotic cells were detected using annexin V-FITC apoptosis detection kit I (Beckman Coulter), according to the manufacturer's instructions. Briefly, MCL cells ($1x10^6$) were cultured in culture medium alone, or with either MS-5 cells or hMSC and harvested on day 5. MCL cells were washed twice with ice-cold phosphate-buffered saline and resuspended ($1x10^6$ cells/mL) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). MCL cells ($1x10^5$) were incubated with annexin V-FITC and propidium iodide for 15 min at room temperature. The cells were analyzed using CXP Cytomics software on an FC500 flow cytometer (Beckman Coulter Immunology, Hialeah, FL, USA).

Cell viability assays

For the determination of cell number and viability, unfractionated MCL cells ($1 \times 10^4/cm^2$) were seeded into T-25 flasks containing either α-MEM (control) alone or with MS-5 or hMSC (90-100% confluency). At the indicated times MCL cells were carefully removed from the monolayer. The monolayer was observed by microscopy to ensure that it was not disrupted). Cell number and viability were measured using trypan blue exclusion on a ViCell Cell Viability Analyzer (Beckman Coulter; Fullerton, CA). The Vicell parameters (size and circularity) were adjusted to discriminate MCL cells from stromal cells. The optimal settings were determined from doping experiments in which different ratios of MCL cells and stromal cells were counted in the Vicell (*data not shown*). In the experiments comparing adherent and non-adherent MCL populations, viability was determined by staining each population with hCD45-Cy7 and 7-AAD followed by flow cytometry.

Cell adhesion assay

For the quantitative determination of adhesive MCL-hMSC interactions, primary hMSC were seeded into 96-well plates and grown for 48 h to confluency. Then $2x10^6$ primary MCL cells were labeled using the Vybrant CFDA SE Cell tracer kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The labeled cells were washed three times in α-MEM and $1x10^5$ cells were added to each well of the 96-well plate. After overnight incubation at 37°C, the non-adherent cells were removed and the plates were washed twice in phosphate-buffered saline. Fluorescence was measured in a fluorescent plate reader (Victor-3; Perkin Elmer) and the number of adherent cells was determined from a standard curve construct-

ed by using the fluorescence of 100 μL aliquots with a known number of labeled cells added to a sister plate treated as above.

Quantification and neutralization of B-cell activating factor

Recombinant human BAFF and recombinant human BAFFR-Fc (decoy receptor) which binds specifically to BAFF and neutralizes its effect, were purchased from Axxora (San Diego, CA, USA). Levels of soluble BAFF in MCL, hMSC, and MCL-hMSC co-culture conditioned α-MEM were quantified in triplicate using commercial enzyme-linked immunosorbent (ELISA) kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. In some experiments used to determine the source of soluble BAFF, MCL or stromal cells were treated with 1% paraformaldehyde before co-culture.

Chemotaxis assay

Chemotaxis experiments were performed using transwells (5 μ pore; Costar, Corning, NY, USA). MCL cells were washed and resuspended in α-MEM with 1% fetal bovine serum. These cells ($5x10^5$) were seeded into the upper chamber and 1 mL of medium containing the indicated concentration of BAFF, CXCL12, CXCL13 and the BAFF decoy receptor (BAFFR-Fc) was added to the bottom chamber. Pertussis toxin (PTX) was used for inhibition of cell chemotaxis. In experiments to determine whether NF-κB plays a role in MCL chemotaxis, MCL cells were infected with either AdGFP (a negative control) or adenovirus containing either dominant-negative IKK1 (IKK1dn) or dominant-negative IKK2 (IKK2dn) (known inhibitors of NF-κB2 and NF-κB1 activation, respectively). After 18 h at 37°C, migrated cells were enumerated by a hemocytometer.

Nuclear factor-κB activation

After the indicated treatment, nuclear extracts were prepared and NF-κB activation was determined by an ELISA kit according to the manufacturer's instruction (Trans-AM NF-κB Family; Active Motif, Carlsbad, CA, USA).

RNA isolation and quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA isolation was performed using the RNAEasy Kit (Qiagen Inc. Valencia, CA, USA) in accordance with the manufacturer's instructions. At least $1x10^6$ cells were harvested from controls and treated cells. Only RNA samples with A260/A280 absorbance ratios between values of 1.8-2.0 were used for quantitative real-time polymerase chain reaction (RT-PCR) experiments. Quantitative PCR experiments were carried out in a total volume of 21 μL on 50-100ng of RNA using Taqman Universal Master Mix (Applied Biosystems). Human β-actin primers (Applied Biosystems) were used as the endogenous control to normalize expression values of mRNA with the ΔΔCT method. BAFF (Hs00198106), IKK1 (Hs00175141) and IKK2 (Hs0023387) probes and primers were purchased from Applied Biosystems.

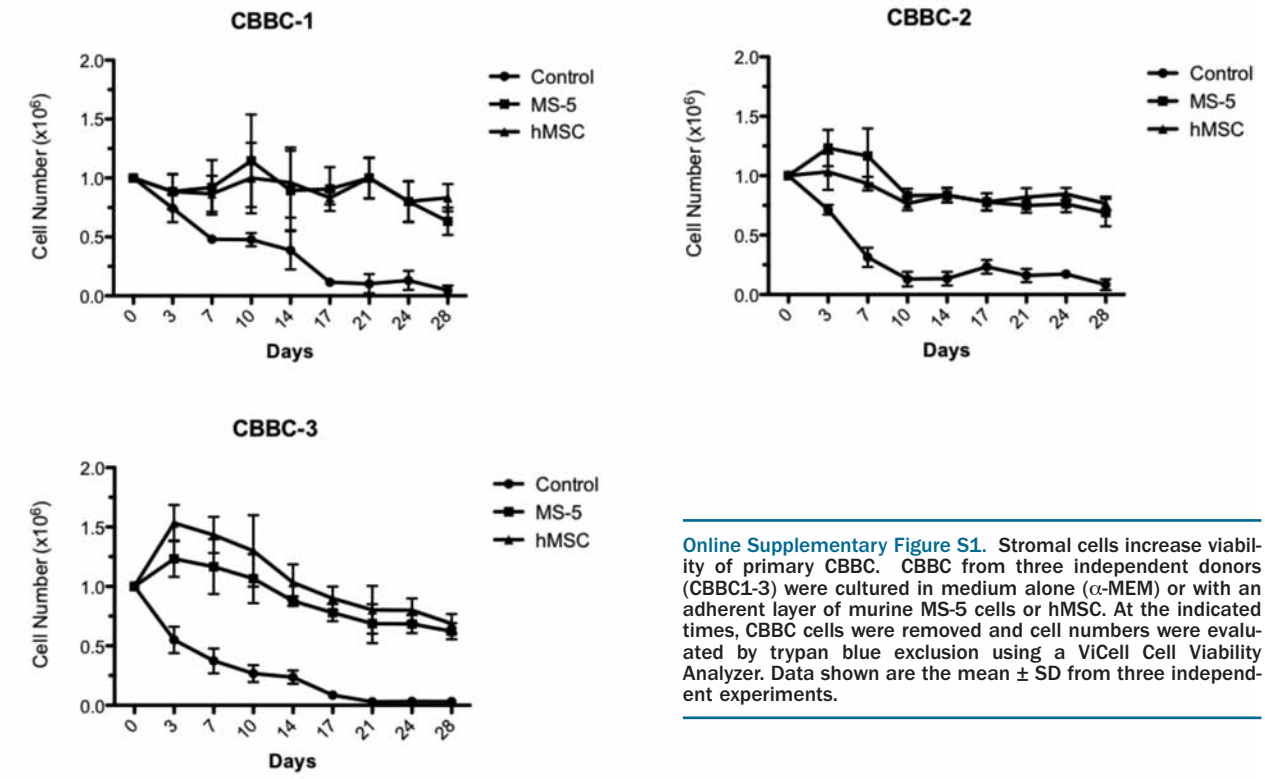
Long-term cultures on human mesenchymal stem/stromal cells

MCL cells and CBBC were plated at a density of $4x10^4$ cells/cm^2 on a pre-established confluent layer of irradiated (25 Gy) hMSC stromal cells (MCL-to-hMSC ratio of 10:1) and maintained at 37°C in 5% CO_2. Once a week, culture populations were halved by carefully removing half of the medium from each culture vessel and adding an equal volume of fresh α-MEM. Every 4 weeks non-adherent cells were removed from the flask and saved. The adherent cells were removed from the flask by the addition of enzyme-free cell disassociation solution (Invitrogen, Carlsbad, CA, USA) followed by two washes in α-MEM. The adherent and non-adherent cells were pooled, counted and MCL cells were then isolated by positive selection isolation using anti-human CD45-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA, USA). Cell purity was then determined by immunophenotyping (CD19, CD3 and CD105). Cells were $CD19^+$ (97±3%), CD105 (3±3%) and no CD3 cells were detected. After MCL isolation the cell concentration was adjusted to $1x10^6$/mL and seeded onto a flask containing a fresh pre-established confluent layer of irradiated (25Gy) hMSC stromal cells (MCL-to-hMSC ratio of 10:1). This was repeated every 4 weeks.

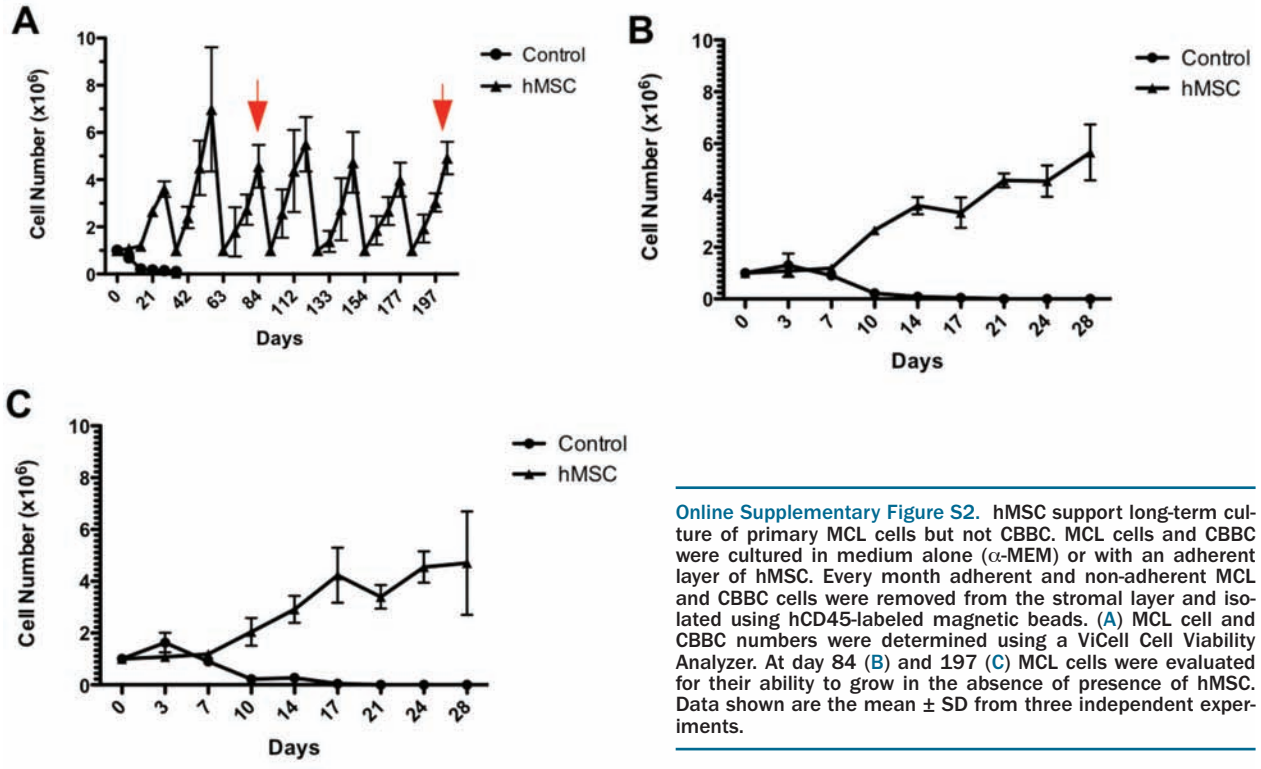
To determine the effects of long-term culture of MCL cells, samples obtained from week 84 and 197 (arrows) cultures were evaluated for immunophenotype (CD3, CD19, CD5, CD23), t(11;14) translocation by fluorescence *in situ* hybridization and their ability to grow in the absence or presence of hMSC as described above.

Drug toxicity assay

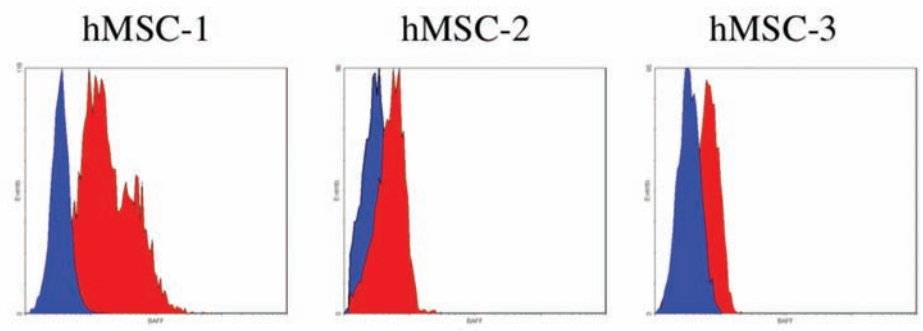
In this series of experiments, MCL cells from six patients were cultured in α-MEM alone or containing doxorubicin (0.33-900 nM), bortezomib (5-160 nM) or fludarabine (5-160 mg/mL). All drugs were obtained from the CINJ research pharmacy. To evaluate the role of BAFF in MCL drug resistance, MCL cells were cultured in either α-MEM (control) or together with hMSC ± 10 μg/mL of the decoy receptor BAFFR-Fc. MCL cell (non-adherent and adherent) viability after 72 h of treatment was determined by staining cells with hCD45-CY7 and 7-AAD followed by flow cytometric analysis. In addition, to help distinguish between MCL cells and stromal cells, we used a lymphocyte gate that excluded large granular stromal cells based on their forward- and side-scatter characteristics.

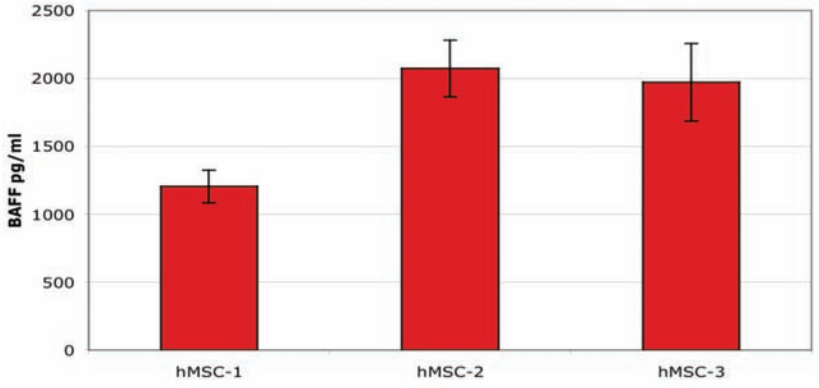


Online Supplementary Figure S1. Stromal cells increase viability of primary CBBC. CBBC from three independent donors (CBBC1-3) were cultured in medium alone (α-MEM) or with an adherent layer of murine MS-5 cells or hMSC. At the indicated times, CBBC cells were removed and cell numbers were evaluated by trypan blue exclusion using a ViCell Cell Viability Analyzer. Data shown are the mean ± SD from three independent experiments.

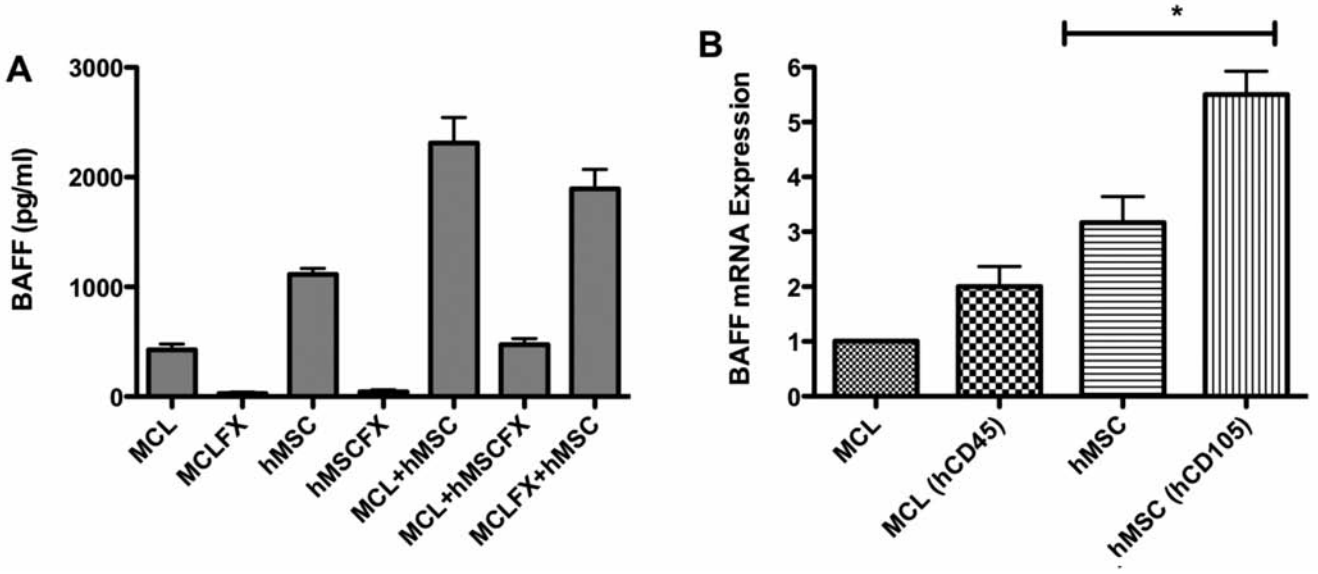


Online Supplementary Figure S2. hMSC support long-term culture of primary MCL cells but not CBBC. MCL cells and CBBC were cultured in medium alone (α-MEM) or with an adherent layer of hMSC. Every month adherent and non-adherent MCL and CBBC cells were removed from the stromal layer and isolated using hCD45-labeled magnetic beads. (A) MCL cell and CBBC numbers were determined using a ViCell Cell Viability Analyzer. At day 84 (B) and 197 (C) MCL cells were evaluated for their ability to grow in the absence of presence of hMSC. Data shown are the mean ± SD from three independent experiments.

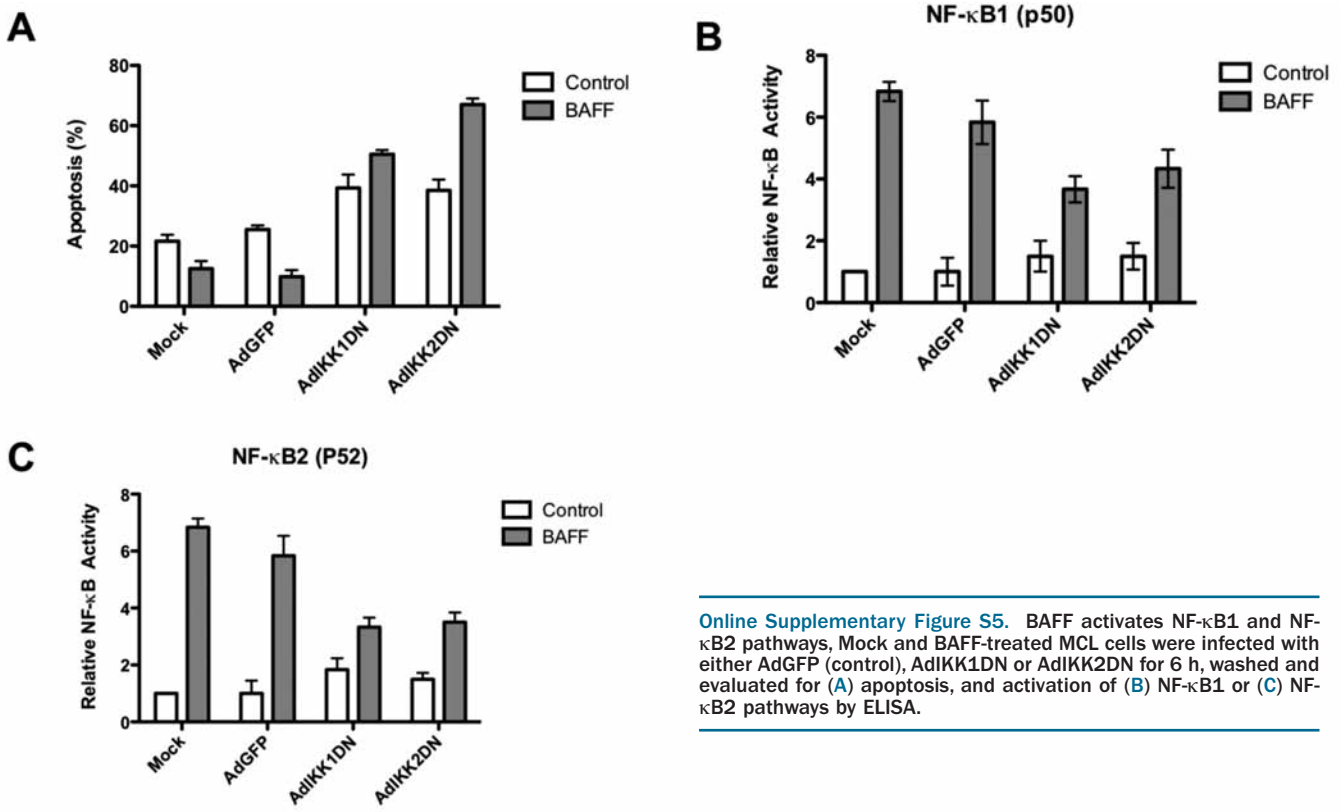




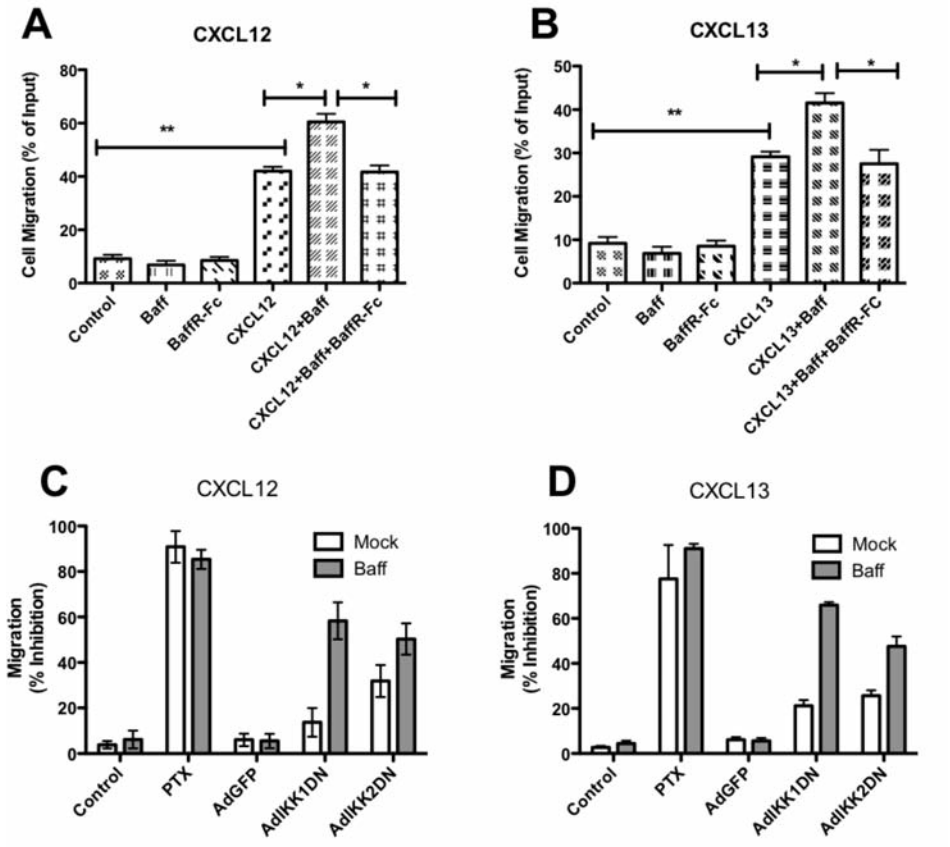
Online Supplementary Figure S3. hMSC produce BAFF in bound and secreted forms. Primary hMSC cells were cultured in α-MEM. After 5 days in culture, cells were removed and stained for surface expression (bound BAFF) with either PE-conjugated isotype control (blue) or mouse anti-human BAFF (red) and analyzed by flow cytometry. The top panel shows a representative histogram-plot from three independent experiments from three healthy donors. Cell-free supernatants collected on day 5 of culture were evaluated for BAFF by ELISA. The concentration of hMSC-secreted BAFF is shown in the bottom panel. Data shown are the mean ± SD of the BAFF concentration from three independent donors.



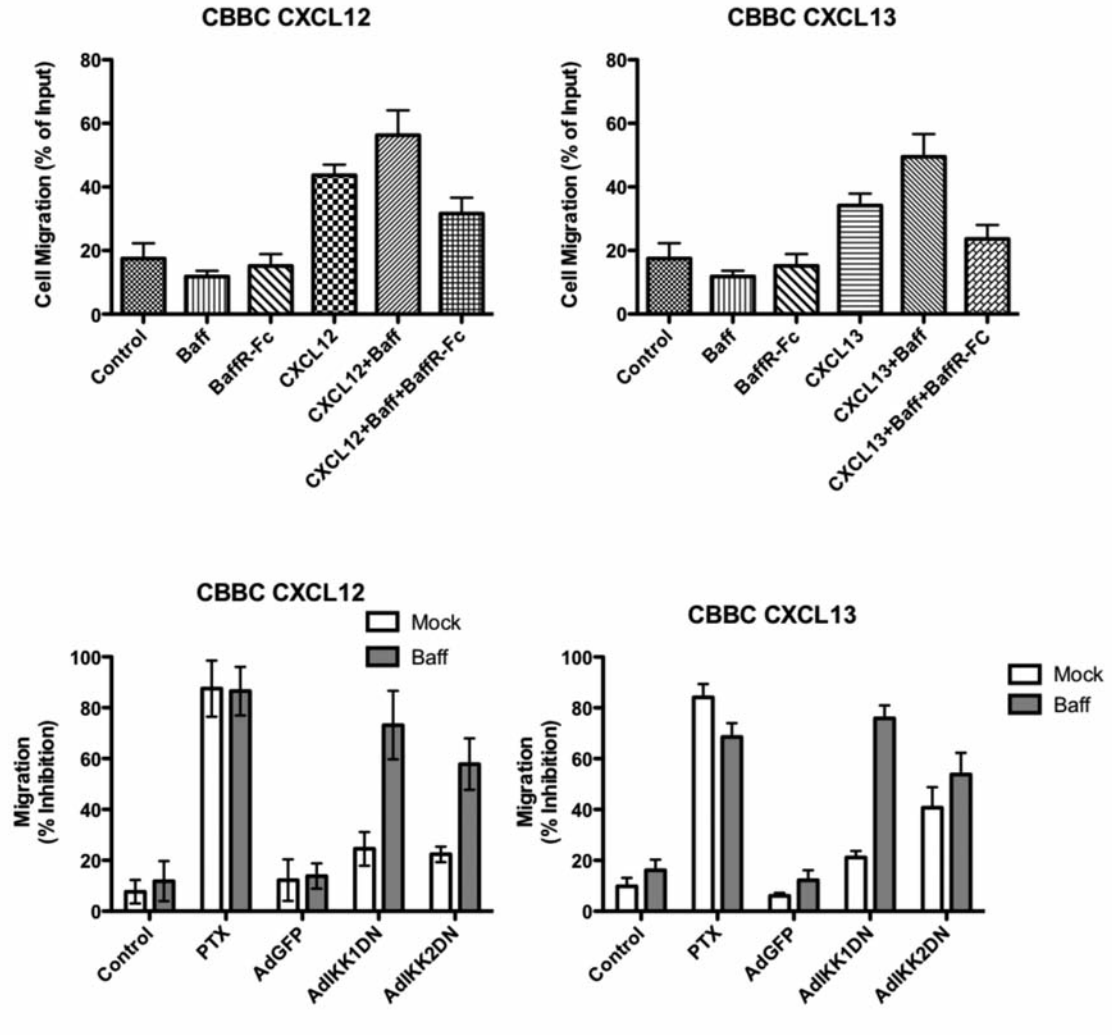
Online Supplementary Figure S4. MCL co-cultured with hMSC induces BAFF secretion and up-regulation of BAFF mRNA. (A) BAFF secretion in culture medium from MCL cells (MCL), hMSC alone (hMSC) or co-cultured (MCL+hMSC) and paraformaldehyde-fixed MCL (MCLFX) or hMSC (hMSCFX). (B) BAFF mRNA measured by real-time quantitative RT-PCR. Fold values were obtained by mRNA fold changes relative to MCL. MCL (MCL alone), hMSC (hMSC alone), and MCL cells after immunomagnetic sorting by hCD45 magnetic beads (hCD45) and hMSC (hCD105) sorting by CD105 magnetic beads. The data shown are the means ± SD of six patients' samples from three independent experiments.



Online Supplementary Figure S5. BAFF activates NF-κB1 and NF-κB2 pathways, Mock and BAFF-treated MCL cells were infected with either AdGFP (control), AdIKK1DN or AdIKK2DN for 6 h, washed and evaluated for (A) apoptosis, and activation of (B) NF-κB1 or (C) NF-κB2 pathways by ELISA.



Online Supplementary Figure S6. BAFF increases the CXCL12- and CXCL13-depedent chemotaxis of MCL cells and is dependent on the canonical and non-canonical NF-κB pathways. MCL cells from four different MCL patients were treated with α-MEM, 100 ng/mL BAFF, 5 μg/mL BAFF-Fc, 250 ng/mL CXCL12 (A), 250 ng/mL CXCL13 (B) or a combination of BAFF and either CXCL12 or CXCL13 ± BAFF-Fc. Mock or BAFF-treated MCL cells were cultured in α-MEM, pertussis toxin (PTX) or infected with either AdGFP, AdIKK1DN or AdIKK2DN. Cells were then evaluated for their ability to specifically migrate to CXCL12 (C) or CXCL13 (D). Data are expressed as the mean percentage ± SD of specific migration from three independent experiments.



Online Supplementary Figure S7. BAFF increases the CXCL12- and CXCL13-dependent chemotaxis of CBBC and is dependent on the canonical and non-canonical NF-κB pathways. CBBC from three different donors were treated with α-MEM, 100 ng/mL BAFF, 5 μg/mL BAFF-Fc, 250 ng/mL CXCL12 (A), 250 ng/mL CXCL13 (B) or a combination of BAFF and either CXCL12 or CXCL13 ± BAFF-Fc. Mock or BAFF-treated CBBC cells were cultured in α-MEM, pertussis toxin (PTX) or infected with either AdGFP, AdIKK1DN or AdIKK2DN. Cells were then evaluated for their ability to specifically migrate to CXCL12 (C) or CXCL13 (D). Data are expressed as the mean percentage ± SD of specific migration from three independent experiments.