Complementarity determining region-independent recognition of a superantigen by B-cell antigen receptors of mantle cell lymphoma

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Supplementary Materials and Methods

RNA isolation from tumor samples: The MCL1 and MCL2 samples were left over bone marrow from diagnostics procedures. The PBMCs were isolated using a Ficoll gradient and the RNA was extracted using the RNeasy Mini Kit (Qiagen). All other samples derived from fresh/frozen lymph node or tumor biopsies (see Table S1) and the RNA was extracted using the mirVana™ miRNA isolation Kit (Thermo Fisher Scientific)

Sequence analysis of MCL-derived immunoglobulin V regions: Two µg of total RNA from patient samples (see Table S1) were transcribed into cDNA using 1 µM oligo(dT) primer and 4 units (U) omniscript reverse transcriptase (Qiagen). After precipitation with ethanol, we used 10 U terminal transferase (NEB) and 1.5 mM dGTP to append a 3'-oligo-G-tail to the cDNA.

The immunoglobulin heavy and light chain genes were amplified by nested PCR with an isotype-specific reverse primer and an oligo-C primer as described by Osterroth et al.[1] (sequences of oligonucleotide primers are shown in Table S4). The amplified products were blunted and cloned into the pJet vector (Invitrogen). At least eight random clones from two independent amplifications were sequenced (Seqlab). Determination of the immunoglobulin heavy and light chain families was done utilizing the IMGT/V-Quest tool.[2,3] A sequence found to be dominant in both amplification rounds was regarded as the tumor clone-derived immunoglobulin sequence.

Cloning and expression of recombinant MCL-derived antibodies: We cloned four different versions of pFastbacDual vector (Invitrogen) with either full length immunoglobulin (Ig) or Fab fragments containing a gamma Ig constant heavy chain as well as kappa or lambda constant Ig light chains, respectively.

Afterwards, the variable regions of the individual tumor Ig genes were cloned into these vectors with respect to the light chain isotype of the Ig.

The vectors were transformed in DH10bac cells to produce bacmids.

The bacmids were transfected into adherent Sf9 cells using the TransIT-reagent (Lonza) and incubated at 27°C. After 72h we collected the supernatant (P1-stock) and infected 1×10^7 Sf9 cells with 400 µl P1 stock for 72h at 27°C in an adherent culture. The resulting P2-stock was frozen at -80°C. A test expression was performed to identify the ideal amount of P2 stock for production. The optimal amount of P2-stock was given to a 300 ml Sf9 suspension culture (1.5×10^6 cells per ml) that was afterwards shaken for 96h at 27°C.

Full length antibodies were isolated from the culture supernatant by affinity purification using protein A sepharose (GE Healthcare). For isolation of Fab fragments, the culture supernatant was dialyzed against PBS overnight at 4°C, followed by affinity purification with Ni-NTA sepharose.

Phage display peptide library screening: A linear 12mer library was purchased from New England Biolabs (NEB). Screenings on MCL immunoglobulins were performed after two-fold negative selection on polyclonal human IgG (Octapharma) according to the manufacturer's instructions. Randomly chosen clones were picked and amplified after four selection rounds and tested for specific binding to MCL-derived Igs (versus IgG) by Enzyme-linked immunosorbent assay (ELISA) in a 96-well plate. The screening antibody as well as the antibodies used for negative selection and a control antibody were coated overnight, blocked with 3% BSA and incubated for one hour with 2×10^7 monoclonal phages. After one hour incubation with HRP-labeled anti-M13 antibody (Sigma-Aldrich), detection buffer was added and the extinction at 405 nm was measured using a Multiscan Spectrum ELISA reader (Thermo Fisher Scientific). DNA of phages showing specific binding was sequenced (Seqlab).

HEp2A-based immunofluorescence: 1×10^5 HEp2A cells were seeded on a coverslip and let grown over night until 70-80% confluency was reached and fixed with 3.7% PFA (Carl Roth) or Methanol (J.T. Baker) and permeabilized using 0.5% Triton X-100 (Carl Roth) if necessary. The cells were blocked with 5% BSA in TBS for one hour at room temperature. All washing steps were performed using TBS. Up to 100 µg/ml of MCL Ig-derived antibodies were incubated over night at 4°C in a wet chamber. For detection of the primary antibody a FITC-labeled anti-human-IgG antibody (Sigma Aldrich) was used. The coverslips were sealed with DAPI-containing mounting medium (Vector Laboratories).

Protein A binding ELISA: Wells were coated with 4 µg Staphylococcus aureus protein A (SpA) (Sigma Aldrich) over night, blocked with 3% BSA in TBS and incubated with 4 µg recombinant Fab fragments diluted in 150 µl 1% BSA in TBS for one hour each. After washing for three times with TBS-T, a HRP-labeled goat anti-human Fab antibody (1: 5000, AbD Serotec) was added for one hour. Afterwards the detection buffer was added and the extinction at 405 nm was measured using a Multiscan Spectrum ELISA reader (Thermo Fisher Scientific).

Establishment of cell lines expressing individual MCL BCRs: The human Burkitt lymphoma cell line Ramos was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were transfected to stably express the murine cationic amino-acid transporter 1 (*Slc7a1*) to make them susceptible for infections with MMLV-based retrovirus particles. Subsequently, surface IgM-negative cells were isolated by repetitive cell sorting.

Ramos cells were plated into a 24 well plate at a concentration of 50,000 cells per well in 500 µL of RPMI medium. The cells were transduced by adding two lentiviral vectors

encoding the Ig variable heavy chain gene segment of the BCR fused to a mouse µ constant region in conjunction with a zeocin resistance gene and the Ig light chain gene with a human lambda constant region in conjunction with a puromycin resistance gene, respectively. Per lentiviral vector, 100 µl of supernatant containing VSV-G pseudotyped viral particle was used. Five days after transduction cells were transferred into a 6-well plate and selection of the double-positive cells (expressing IgH and IgL) was started by adding 1 µg/ml puromycin (Sigma Aldrich) and 20 µg/ml zeocin (Invitrogen).

Membrane expression of recombinant Ig heavy and light chains was confirmed by flow cytometry using a FITC-labeled anti-mouse IgM antibody (BD Bioscience) and PE-labeled anti-human lambda antibody (Dako). To exclude the possibility of residual endogenous BCR expression, we also stained with FITC-labelled anti-human IgM antibody (Dako). A FACSCalibur (BD Biosciences) was used for analysis.

Ca^{2+} flux measurement: The protocol was adapted from Schepers et al..[4] Two PBS solutions with and without Ca^{2+} (GIBCO) were supplemented with 25 mM HEPES and 1 mM sodium-pyruvat (GIBCO). On average, 3×10^6 transduced and untransduced Ramos cells were washed with PBS+Ca^{2+}(0.9 mM Ca^{2+}) and loaded with 5 µM Fluo-4-AM (Invitrogen) for 30 min at room temperature in the dark. After washing with PBS without Ca^{2+}, the cells were resuspended in 1 ml PBS+Ca^{2+}. The fluorescence intensity measurements of Fluo-4-loaded cells were performed using a FACS Calibur (BD Bioscience) and the BD CellQuest Pro software (version 5.2.1). For BCR stimulation, 5 µg/ml SpA (Sigma Aldrich) or 1 µg/ml goat anti-human Fab antibody (AbD-Serotec) was used. Analysis was done using FlowJo software (version 8.7).

Western Blot: Cell lysates were generated using RIPA buffer. 10 µg of the RIPA lysates or 2 µg of the IgG were separated on 10 % SDS-polyacrylamide gels, transferred to a PVDF

membrane and blocked with 5 % skim milk in TBS-T. Goat-anti-human-IgG-(Caltag), goat-anti-human-IgM- (Southern Biotec) and mouse-anti-beta actin- (Sigma Aldrich) antibodies were used as primary antibodies. HRP-labelled anti-goat-IgG- (Santa Cruz) or HRP-labelled anti-mouse-IgG- (Santa Cruz) antibodies were used for detection, respectively.

Far-Western blot analysis: 25 µg SpA (Sigma Aldrich), anti-mouse-IgM- (eBioscience) or anti-Fab (AbD Serotec) antibodies dissolved in 100 µl TBE-buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) were coated per well in a 6-well plate (Greiner Bio One) for 20 min at 37°C. Per well 1×10^7 cells of the transduced or untransduced Ramos cells were directly added, incubated for 2h at 37°C , pelleted and lysed in KLB-lysis buffer (150 mM NaCl, 25 mM TrisHCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 10% glycerol, 10 mM sodium pyrophosphate, 1 mM sodium ortho-vanadate, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 0.2 mg/mL aprotinin, 10 mM sodium fluoride and 0.1 mM freshly prepared sodium pervanadate). 15 µg of protein extract were separated on 4-12% SDS-PAGE gradient gels (Invitrogen), transferred to a PVDF-membrane and blocked in 10% skim milk in TBST-buffer at 4°C overnight. Subsequently, membranes were probed with 1 µg/mL of biotinylated GRB2 SH2 domain precomplexed with streptavidin-horseradish peroxidase (Pierce, USA) as previously described.[5]

Supplementary Figures

Table S1: Origin of MCL tumor samples.

Sample	acquired from
MCL4	lymph node
MCL21	tumor
MCL32	lymph node
MCL23	lymph node
MCL1	bone marrow
MCL8	lymph node
MCL11	base of the mouth (tumor)
MCL20	lymph node
MCL28	lymph node
MCL2	bone marrow
MCL22	spleen (tumor)
MCL13	gall bladder (tumor)
MCL5	spleen (tumor)
MCL31	lymph node
MCL16	lymph node
MCL19	lymph node
MCL24	lymph node
MCL29	lymph node
MCL25	lymph node
MCL14	lymph node
MCL30	lymph node
MCL27	base of the tongue (tumor)
MCL12	lymph node
MCL18	lymph node

Table S2: Enriched phage peptide sequences after four selection rounds on recombinant MCL BCR-derived immunoglobulins.

Sample	V gene seqment family	Enriched peptide sequences
MCL4	IGHV1-8	**NCMMVKPTLIVC**
MCL23	IGHV3-9	**No enrichment**
MCL11	IGHV3-21	**No enrichment**
MCL2	IGHV3-23	**No enrichment**
MCL22	IGHV3-30	**SLFYTACNPKVC** **CNHKCAELVTSA**
MCL5	IGHV3-49	**No enrichment**
MCL19	IGHV4-34	**No enrichment**
MCL24	IGHV4-34	**No enrichment**
MCL27	IGHV4-59	**No enrichment**
MCL12	IGHV5-51	**No enrichment**
MCL18	IGHV5-51	**WKGQYVSNIPLL** **YSGDPKWRWLPQ** **YDLKWEYVLKPP** **DNKWQHVINYSL**

Note: Immunoglobulins harboring **IGHV**3 family segments are presented with gray background.

Table S3: Overview of CLL and FL gene families and CDR3 regions from follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL) samples used for motif determination as well as SpA ELISA.

Sample	V gene segment family	HCDR3	V-region identity in %
FL6	IGHV3-11	ARDVGGDHDHRFDP	87,85
FL10	IGHV 3-11	VIGSVATIFAN	80,21
FL8	IGHV 3-11	VKSRYNGNYYFDY	93,06
FL101	IGHV 3-15	TTLNNSAWDN	84,35
FL11	IGHV3-23	VKNETDYYKMDF	81,25
FL5	IGHV 3-53	ARGRMGSNDSFDF	87,72
CLL149	IGHV 3-7	ARAGSYYDFWSGQITGGMDV	94,44
CLL172	IGHV 3-7	AREARIVGATTIDY	95,49
CLL024	IGHV 3-9	AKGGARWIQLTVRSYYYYYGMDV	100
CLL83	IGHV 3-21	ARDANNMDV	95,49
CLL173	IGHV 3-23	AKAHRDYSSSSVDY	93,06
CLL003	IGHV 3-30	AGVVEMATIGGVFSGMDV	100
CLL145	IGHV 3-30	AKGAYSGYDLAIMITFGGVIVSGGFDY	99,31
CLL167	IGHV 4-31	ARVGSGSPYYYGVDV	99,66

Note: Immunoglobulins harboring IGHV3 family segments are presented with gray background.

Table S4: Primer sequences used for amplification of immunoglobulin variable regions.

Primer name	Sequence
BaPpC (fw)	5'- CTCTGCAGGATCCACGACCCCCCCCCCCCC
BaP (fw nested)	5'- TCTGCAGGATCCACGACC
IgM rev	5'- CTCTCAGGACTGATGGGAAGCC
IgM rev nested	5'- CAGGAGACGAGGGGGAAAAG
Kappa rev	5'- CTGATGGGTGACTTCGCAG
Kappa rev nested	5'- GCTCATCAGATGGCGGGAAG
Lambda rev	5'- CGTGACCTGGCAGCTGTAG
Lambda rev nested	5'- CAGAGGAGGGTGGGAACAG

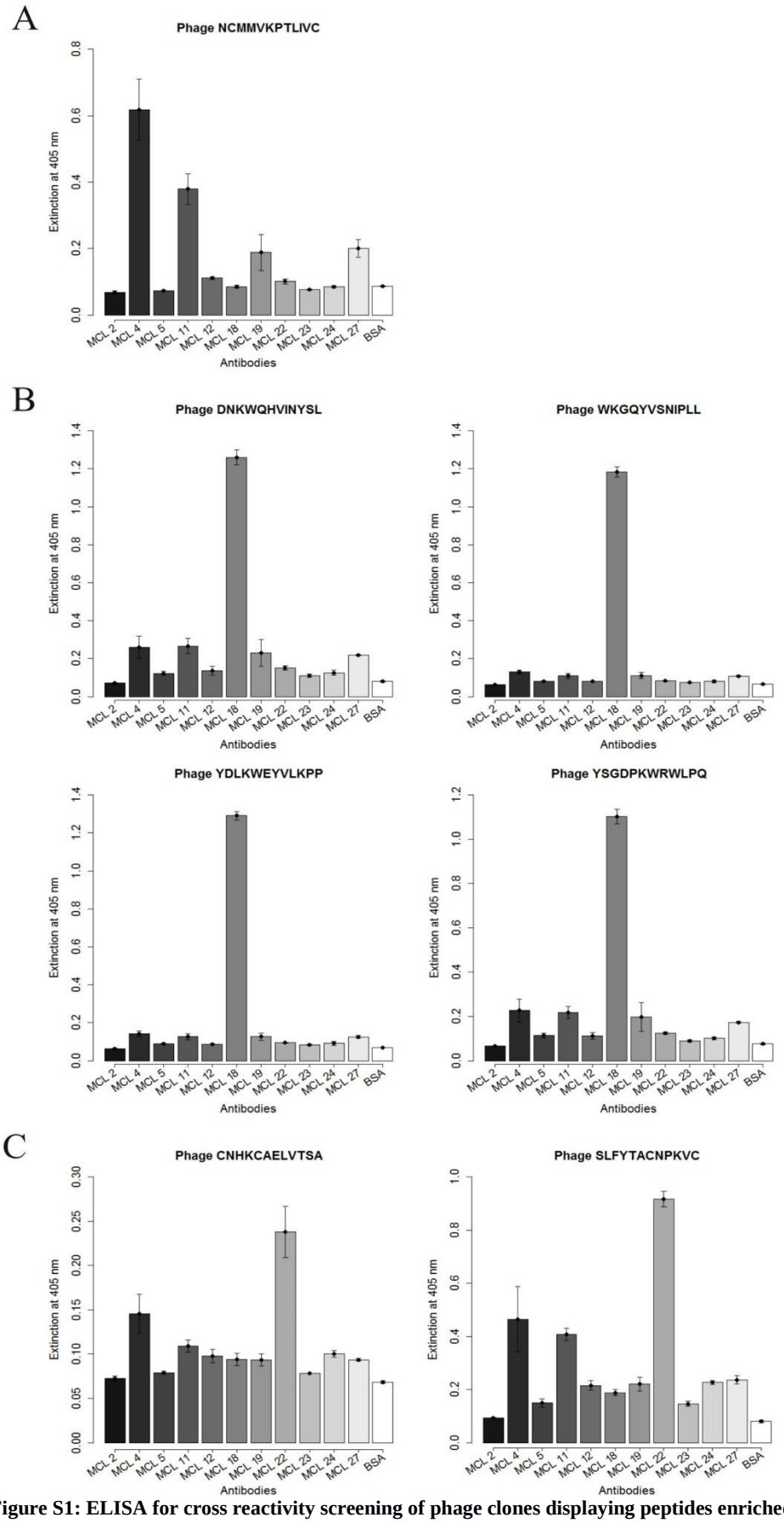

Figure S1: ELISA for cross reactivity screening of phage clones displaying peptides enriched during random peptide phage library selections. A: ELISA with phage peptide selected on MCL4 immunoglobulin. B: ELISAs of phage peptides enriched on MCL18 selection and C: ELISA on phage peptides enriched on MCL22 selection. Note that all phages strongly bound to the immunoglobulins used for selection but only minimally cross reacted with other MCL BCR-derived immunoglobulins.

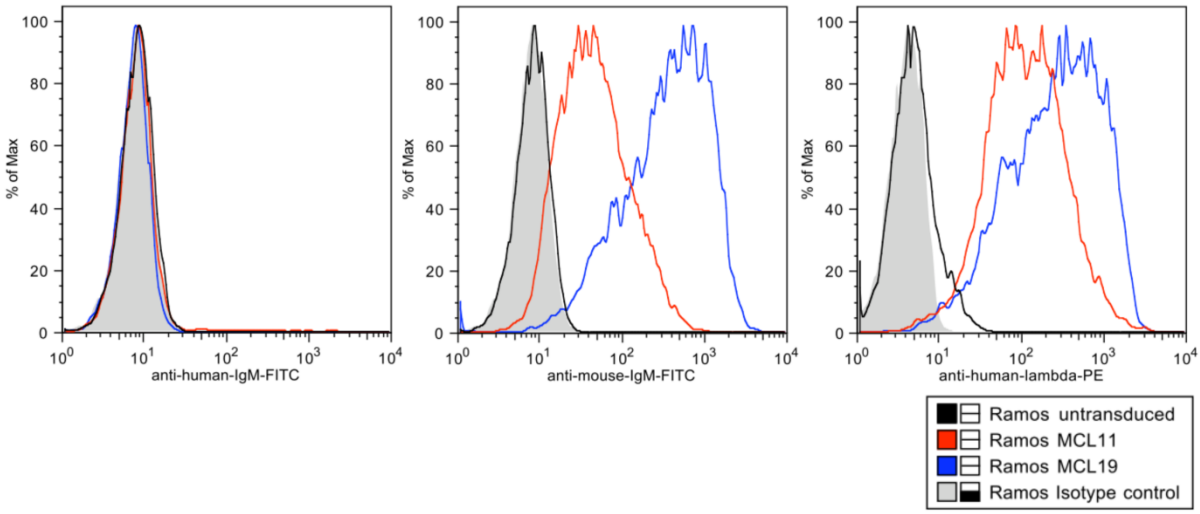

Figure S2: FACS analysis of Ramos cells transduced with recombinant MCL-derived immunoglobulin H and L chain genes. Cells were stained with a FITC-labelled anti-human IgM- (left), a FITC-labelled anti-mouse IgM- (center) or a PE-labelled anti-human-lambda (right)-antibody. No residual endogenous human-IgM signal was detected in all samples and the transduction with MCL-derived Igs into Ig-negative Ramos cells was successful (red and blue lines).

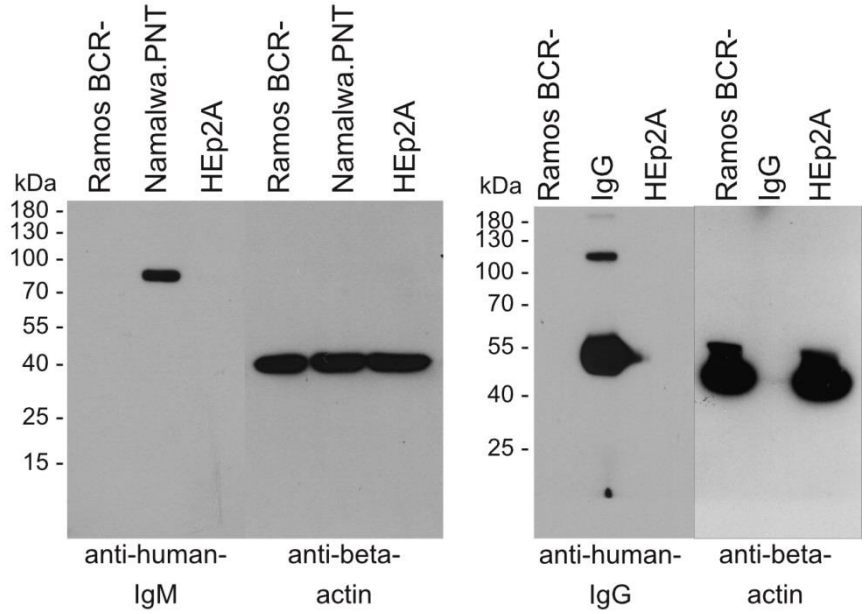

Figure S3: Western Blot with RIPA cell lysate of sorted membrane Ig negative Ramos cells. No residual endogenous human IgM- or IgG-expression was left in Ramos cells, which were sorted for Ig negativity. Namalwa.PNT and recombinant IgG served as positive control for the anti-human-IgM and anti-human-IgG antibody, respectively. HEp2A cell lysate was used as negative control and beta-actin served as loading control.

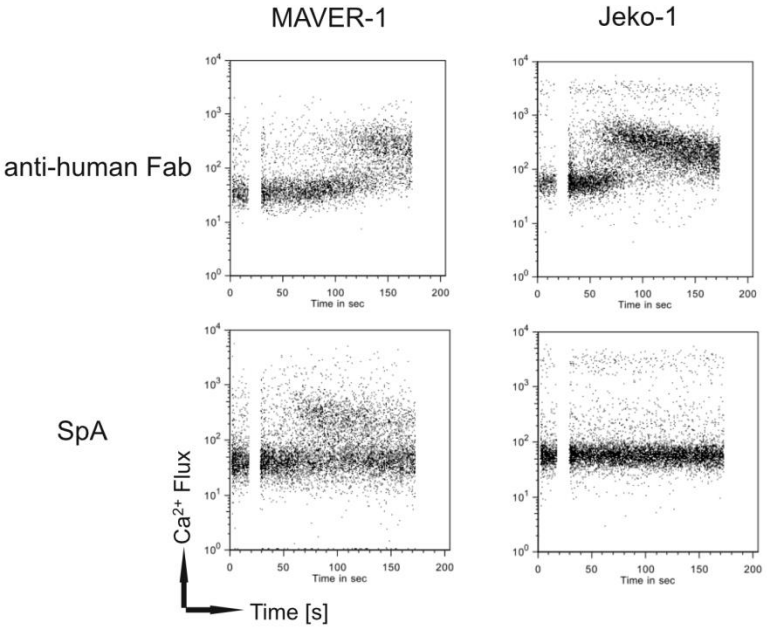

Figure S4: Ca Flux with the SpA motif positive MAVER-1 and the SpA motif negative Jeko-1 MCL cell lines. Cells were loaded with Fluo-4 and induced with 1 µg/ml anti-human-Fab antibodies or 5 µg/ml SpA.

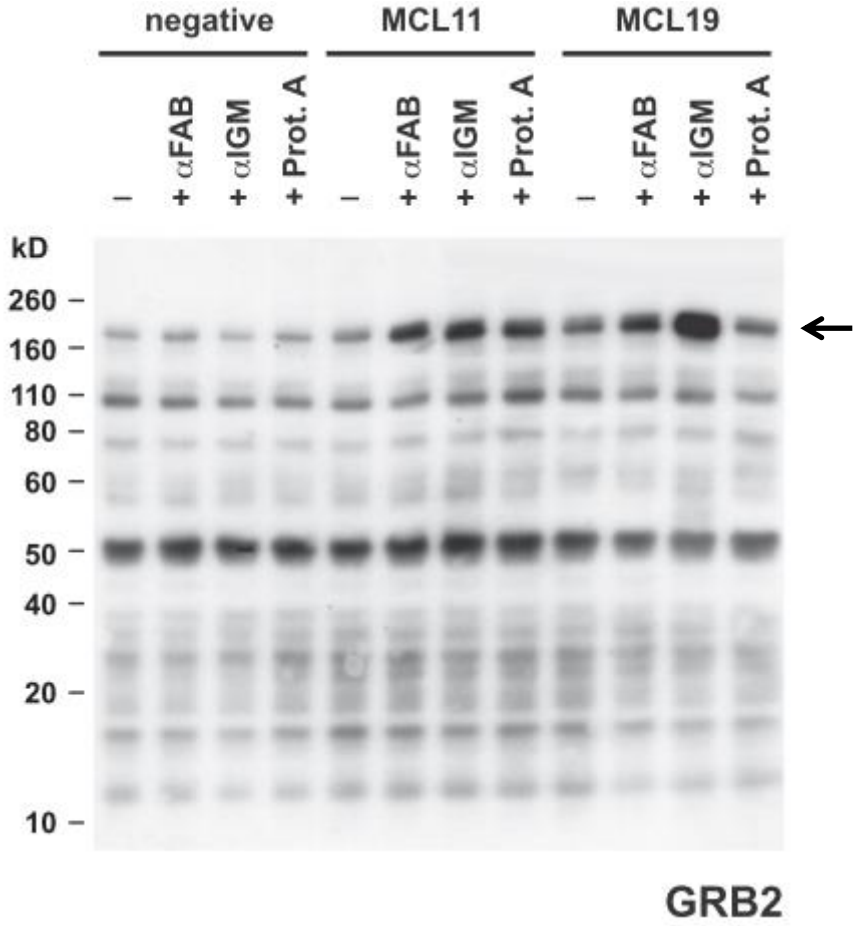

Figure S5: Far-Western Blot for detection of differential tyrosine phosphorylation in stimulated and unstimulated Ramos cells using GRB2 SH2 domain as a phosphoprobe. MCL-derived BCR expressing and untransduced Ramos cells were incubated for 2 h with anti-human-Fab antibody, anti-mouse-IgM antibody or protein A (SpA), respectively. The cell lysates were separated by SDS-PAGE and the phosphorylation was probed with the GRB2 SH2 domain. Note the increased signal intensity in MCL11 expressing Ramos cells stimulated with SpA in comparison to the untreated and MCL19 expressing cells, particularly for the phosphoprotein band of approximately 180 kDa (arrow).

Supplementary References:

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