FcγRIIb-BCR coligation inhibits B-cell receptor signaling in chronic lymphocytic leukemia

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SUPPLEMENTARY FIGURES.

Supplementary Figure 1. Heterogeneous BCR responses among CLL patient samples. (A) Immunoblots for 3 representative CLL patient's samples that responded to BCR ligation with an increase of p-AKT or p-ERK. Purified CLL cells were exposed to $F(ab')_2$ anti-human IgM (10µg/mL) for 5 minutes before being lysed and immunoblotted for p-AKT and p-ERK. Equal loading was checked by immunoblotting with GAPDH. (B) Calcium kinetics for a representative CLL patient's sample that responded with an increase of calcium flux after BCR ligation. Purified CLL cells were loaded with Indo1 (4µM) and the baseline Indo1 ratio was acquired for 60 seconds prior to the addition of $F(ab')_2$ anti-human IgM. (C) Immunoblots for 3 representative CLL patient samples that remained anergic upon BCR ligation. (D) Calcium kinetics for a representative CLL sample that did not exhibit any variation in calcium flux upon BCR ligation. F: $F(ab')_2$ anti-human IgM pAbs.

Supplementary Figure 2. Molecular effects of BCR ligation or FcγRIIb-BCR co-ligation in normal B-cells. Immunoblots for two representative healthy donor's samples (A) and graphic representation of relative p-ITIM (B), p-SHIP (C), p-AKT (D) and p-ERK (E) expression in normal B-cells from healthy donors (n=6). Purified B-cells from healthy donors were exposed to $F(ab')_2$ anti-human IgM (10µg/mL), whole anti-human IgM (15 µg/mL) or specific anti-FcγRIIb monoclonal antibody (2B6) (1µg/mL) for 5 minutes before being lysed and immunoblotted for p-ITIM domain of FcγRIIb, p-SHIP, p-AKT and p-ERK. Equal loading was checked by immunoblotting with GAPDH. Bar charts show mean values and standard deviation. *P*-values were calculated using two tail paired t-test (*$p<0.05$, **$p<0.01$). F: $F(ab')_2$ anti-human IgM pAbs; W: whole anti-human IgM pAbs; 2B6: specific anti-human FcγRIIb monoclonal antibody.

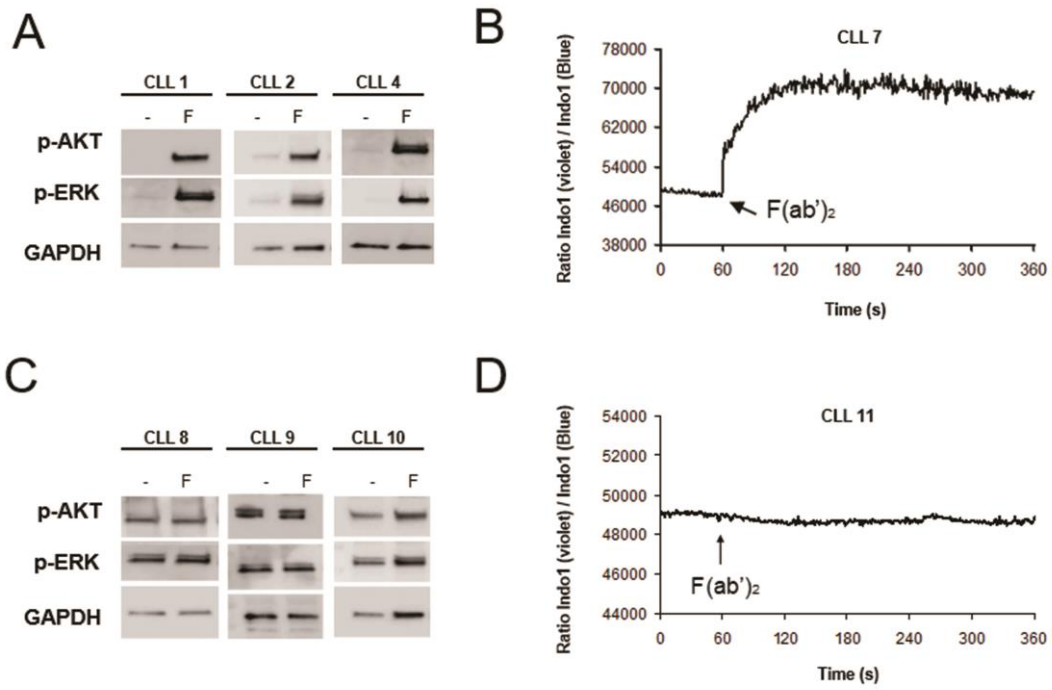
Supplementary Figure 3. Involvement of SHIP in the inhibitory action of FcγRIIb-BCR colligation in normal-B cells. (A) Purified B-cells were pretreated with 10 µM of 3AC or EtOH during 1h before being exposed to the indicated stimuli for 5 minutes. Afterwards, cells were lysed and immunoblotted for p-SHIP, p-AKT, and p-ERK. Equal loading was checked by immunoblotting with GAPDH. Immunoblots for a representative healthy donor (n=3). B) Relative SHIP expression in B-cells transfected with siRNA-control or siRNA-SHIP-1 (n=3). C) Relative AKT and ERK phosphorylation in SHIP-1 siRNA transfected (n=3) and non-transfected (n=6) B-cells. F(ab')2 fragments of anti-human IgM (10µg/ml) or whole anti-human IgM (15 µg/ml) polyclonal antibodies (pAbs) were used to ligate the BCR alone or to co-ligate it with FcγRIIb, respectively. Bar charts show mean values and standard deviation. *P*-values were calculated using two tail paired t-test (*p<0.05, **p<0.01). F: $F(ab')_2$ anti-human IgM pAbs; W: whole anti-human IgM pAbs; 2B6: specific anti-human FcγRIIb monoclonal antibody.

Supplementary Figure 4. Activation, apoptosis and proliferation of normal B-cells upon BCR ligation or FcγRIIb-BCR co-ligation. (A) Calcium kinetics for a representative heathy donor sample. Purified normal B-cells were loaded with Indo1 (4µM) and the baseline Indo1 ratio was acquired for 60 seconds prior to the addition of the different stimuli. Box plots show the percentage of (B) $CD69^+$ cells proportion, (C) cell apoptosis and (D) cell proliferation (n=10). Purified B-cells from healthy donors were exposed to the indicated stimuli for 48h or 72 h and then analyzed by flow cytometry for Annexin V and TO-PRO®-3 (Annexin V^+ cells were considered apoptotic cells), CD69 expression on viable cells, or EdU incorporation using the Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit. *P*-values were calculated using two tail

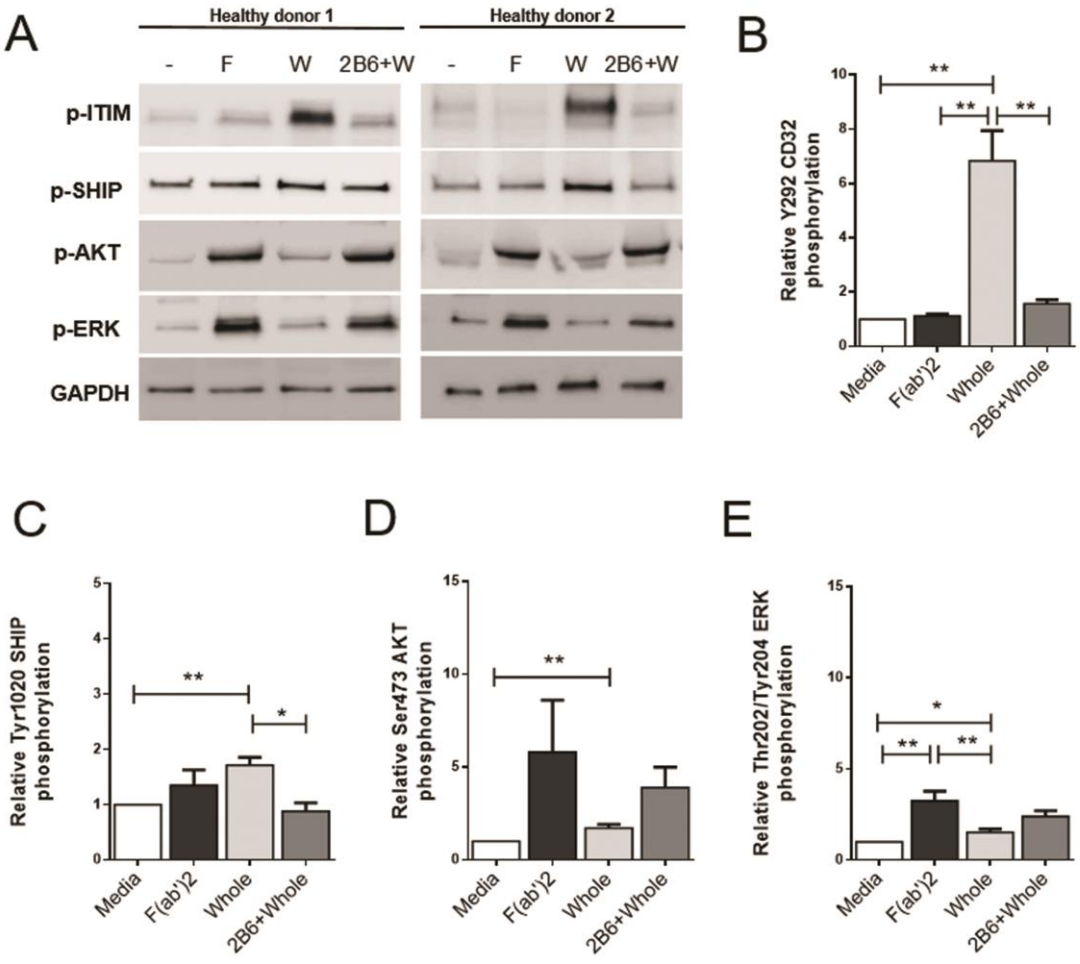
paired t-test (*p<0.05, **p<0.01, ***<0.001). F: F(ab')$_2$ anti-human IgM pAbs; W: whole anti-human IgM pAbs; 2B6: specific anti-human FcγRIIb monoclonal antibody.

Supplementary Figure 5. Apoptosis and proliferation of CLL cells upon BCR ligation or FcγRIIb-BCR co-ligation. Dot plots for a representative CLL patient's sample in which purified CLL cells were exposed to F(ab')$_2$ anti-human IgM (10µg/mL) and whole anti-human IgM (15 µg/mL) polyclonal antibodies (pAbs) or specific anti-human FcγRIIb monoclonal antibody (2B6) (1µg/mL) for 48h or 72h. (A) Apoptosis was analyzed by flow cytometry using Annexin V and TO-PRO®-3 (Annexin V$^+$ cells were considered apoptotic cells). (B) Proliferation was assessed by EdU incorporation using the Click-iT^{TM} EdU Alexa Fluor^{TM} 488 Flow Cytometry Assay Kit.

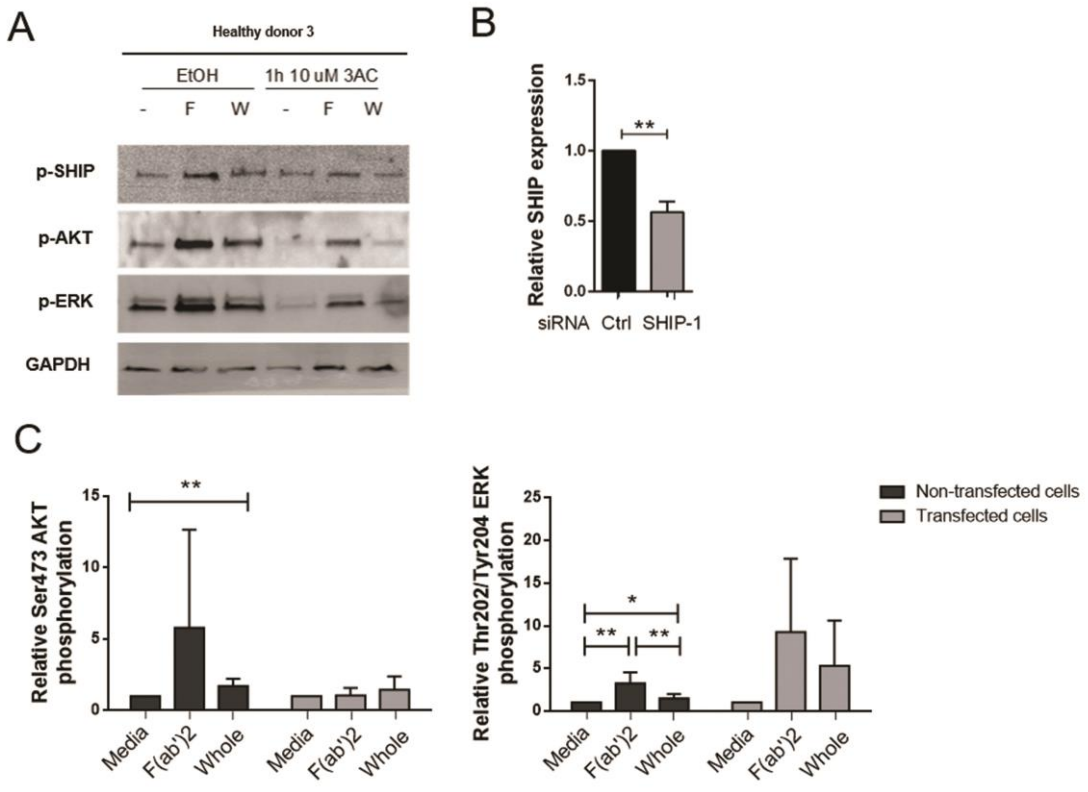
Supplementary Figure 1.



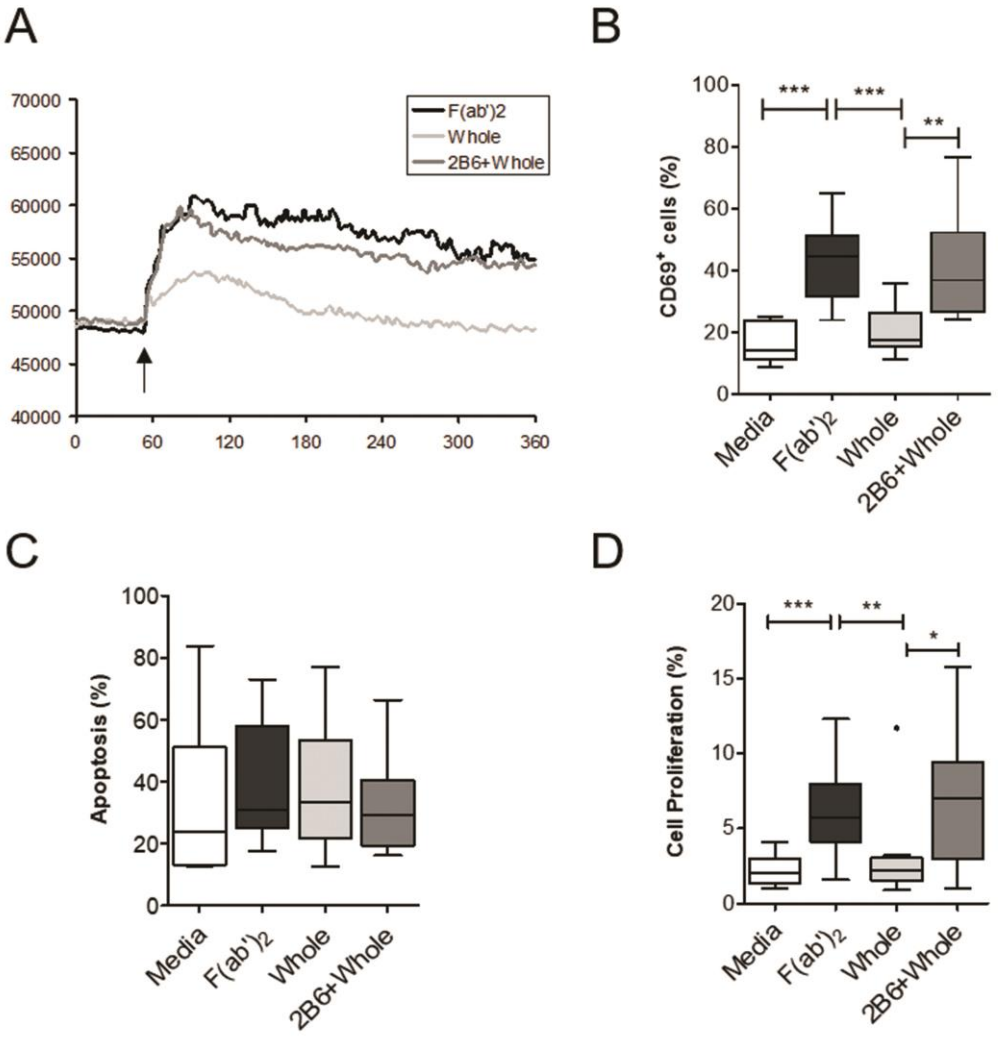
Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.



Supplementary Figure 5.