

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

B-cell receptor (BCR) signaling plays a key role in the pathogenesis of chronic lymphocytic leukemia (CLL) cells, which exhibit higher intrinsic BCR activity than normal B cells.¹ In normal B cells, BCR signaling can be inhibited by the low affinity receptor FcγRIIb (CD32b). This receptor exerts its negative effect when coligated with the BCR by cognate immune complexes (IC) and contributes to maintain peripheral tolerance and to shape the B-cell repertoire. FcγRIIb-BCR coligation counteracts BCR signaling by inducing phosphorylation of the intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) of FcγRIIb, and the subsequent recruitment of (SH2)-containing inositol phosphatases (SHIP), which inhibits Ca⁺⁺ flux and cell proliferation.^{2,3} Considering the major role of BCR in the pathogenesis of CLL and the activated nature of the leukemic cells,¹ we aimed at investigating whether FcγRIIb could regulate BCR signaling in this malignancy. For that purpose, we compared molecular and functional effects of BCR ligation and BCR-FcγRIIb cross-linking on purified B-CLL cells and normal B cells after exposure to equimolar concentrations of F(ab')₂ anti-human immunoglobulin M (IgM) (10 μg/mL) or whole anti-human IgM (15 μg/mL) for 5 minutes.

In agreement with previous reports,^{4,5} BCR ligation induced highly heterogeneous responses on CLL cells. Twelve of 22 (55%) CLL samples responded to BCR

binding with an increase of phospho-AKT (p-AKT) and/or phospho-ERK (p-ERK) levels, as well as an increase of intracellular Ca⁺⁺ influx (*Online Supplementary Figure S1*). BCR responsiveness was neither significantly associated with FcγRIIb expression ($P=0.053$) nor with IgM expression ($P=0.307$).

As a subset of CLL patients show high levels of circulating IC and express poly-reactive BCR, it is reasonable to expect CLL cell interactions with a variety of IC, which could activate FcγRIIb inhibitory signaling. In murine B cells, recruitment of SHIP through FcγRIIb-BCR coligation leads to inactivation of anti-apoptotic kinase AKT^{7,8} and/or inhibition of ERK.^{9,10} Nevertheless, the inhibitory effect of FcγRIIb on BCR signaling has been only cursorily investigated in CLL.^{11,12} Gamberale *et al.*¹¹ suggested that FcγRIIb may inhibit BCR signaling in CLL cells by reducing ERK phosphorylation. Here, similarly to what we observed in normal B cells (*Online Supplementary Figure S2A-C*), the crosslinking of FcγRIIb and BCR in leukemic B cells from BCR-responsive patients induced a significant increase in tyrosine phosphorylation of the ITIM domain of FcγRIIb as well as SHIP phosphorylation (p-SHIP) and translocation to the membrane (Figure 1). In addition, as observed in normal B cells, FcγRIIb-BCR coligation blocked the BCR-induced increase of p-AKT and p-ERK levels (Figure 1C). These inhibitory effects of FcγRIIb were reverted when coligation with BCR was abrogated by adding the specific anti-FcγRIIb monoclonal antibody 2B6 to the whole anti-IgM pAb. Interestingly, ligation of BCR alone also induced a significant increase

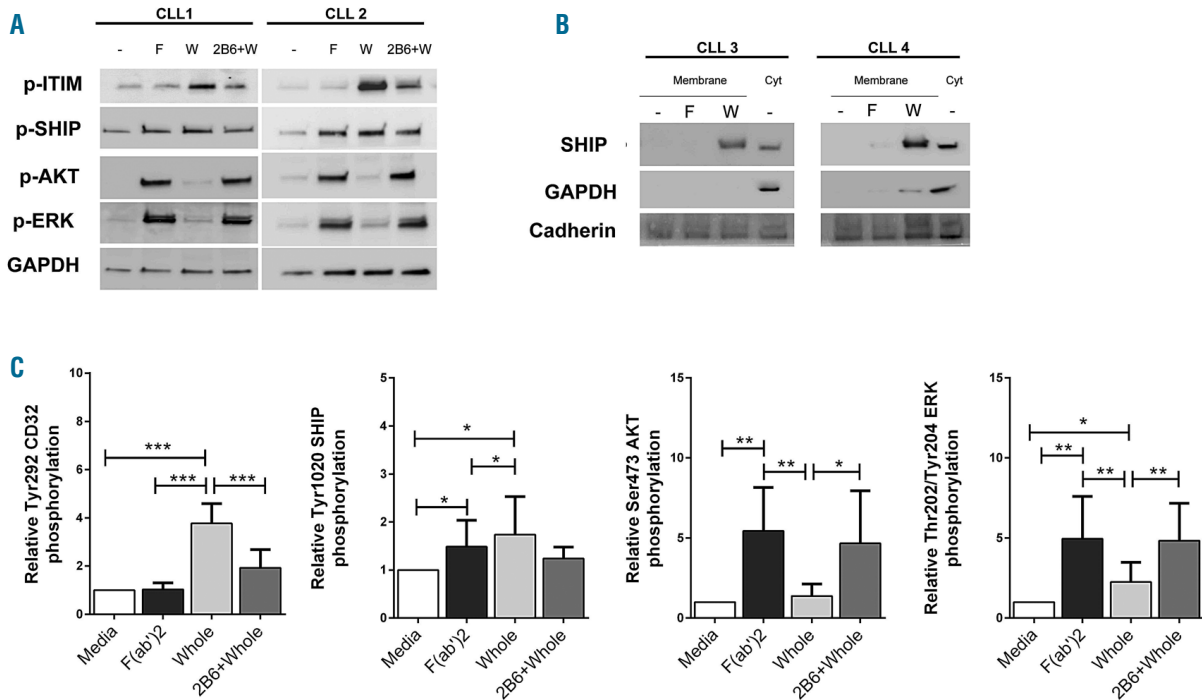


Figure 1. Molecular effects of B-cell receptor (BCR) ligation or FcγRIIb-BCR co-ligation in chronic lymphocytic leukemia cells with responsive BCR. (A) Immunoblot for two representative chronic lymphocytic leukemia (CLL) patient's samples that responded to BCR ligation. Purified B cells from CLL patients were exposed to F(ab')₂ anti-human immunoglobulin M (IgM) (10 μg/mL) and whole anti-human IgM (15 μg/mL) polyclonal antibodies (pAb) or specific anti-human FcγRIIb monoclonal antibody (2B6) (1 μg/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 (n=9). (B) Immunoblot analyses of SHIP in membrane fraction extracts of CLL cells from two representative patient's samples. The remaining extract containing cytosolic fraction of non-treated cells was used as control. The absence of GAPDH expression indicated no cytosolic contamination of the membrane fraction. Cadherin was used as a membrane fraction marker, as well as a control for protein loading. (C) Bar charts show mean values and standard deviation for the relative expression of p-ITIM, p-SHIP, p-AKT and p-ERK in CLL patients with responsive BCR (n=9). P-values were calculated using two tail paired t-test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). F: F(ab')₂ anti-human IgM pAb; W: whole anti-human IgM pAb; 2B6: specific anti-human FcγRIIb monoclonal antibody.

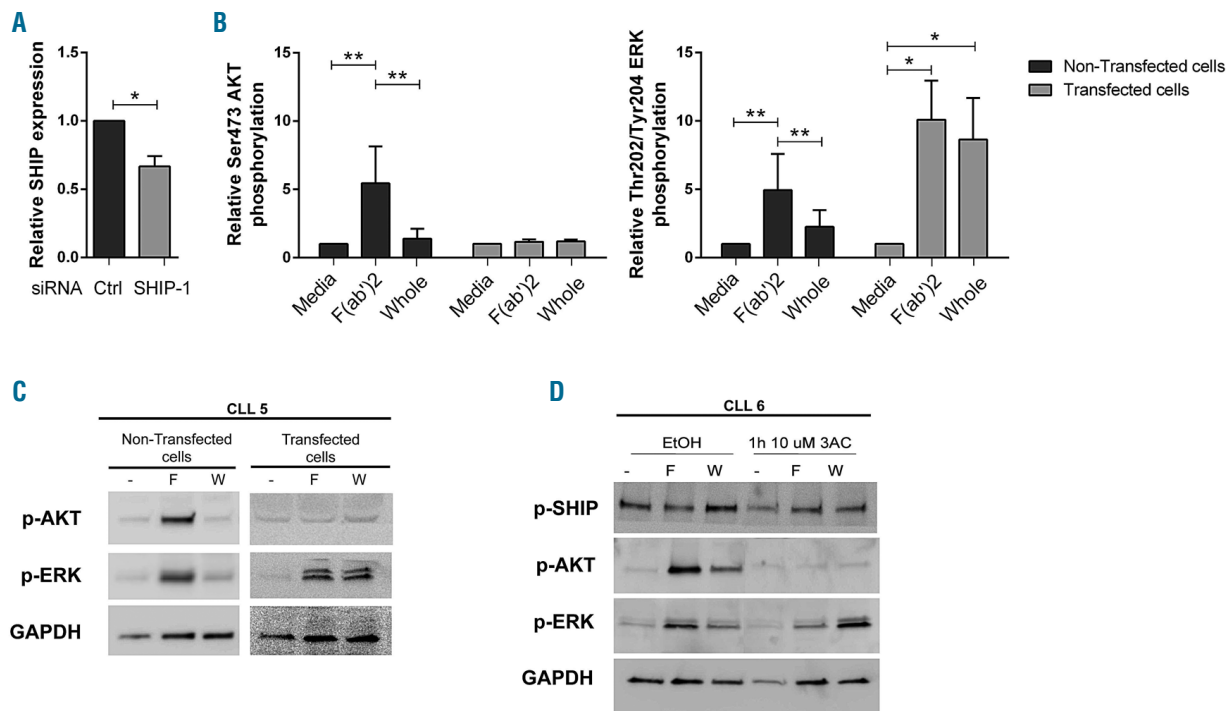


Figure 2. Involvement of SHIP in the inhibitory effect of Fc γ RIIb-B-cell receptor coligation on chronic lymphocytic leukemia cells. (A) Relative SHIP expression in chronic lymphocytic leukemia (CLL) B cells transfected with short interfering RNA (siRNA) *SHIP-1* (n=3). (B) Relative AKT and ERK phosphorylation in *SHIP-1* siRNA transfected (n=3) and non-transfected (n=9) CLL B cells. (C) Immunoblot for a representative CLL patient's sample transfected or not transfected with siRNA *SHIP-1*. (D) Immunoblot for a representative CLL patient's sample exposed to 10 μ M of 3AC or EtOH. F(ab'), fragments of anti-human immunoglobulin M (IgM) (10 μ g/mL) or whole anti-human IgM (15 μ g/mL) polyclonal antibodies (pAb) were used to ligate the BCR alone or to coligate it with Fc γ RIIb, respectively. Cells were lysed and immunoblotted for SHIP, p-SHIP, p-AKT and p-ERK. Equal loading was checked by immunoblotting with GAPDH. Mean values, standard deviation and statistical significance between data were determined by two-tailed paired t-test (* P <0.05, ** P <0.01). F: F(ab'), anti-human IgM pAb; W: whole anti-human IgM pAb; 2B6: specific anti-human Fc γ RIIb monoclonal antibody.

of p-SHIP levels, as reported before^{13,14} indicating that SHIP can also function independently of Fc γ RIIb.

We also studied the potential involvement of SHIP in the inhibition of p-AKT and/or p-ERK induced by Fc γ RIIb-BCR cocrosslinking. For that purpose, we inhibited SHIP using two different approaches; cell transfection with a specific SHIP-1 short interfering RNA (siRNA) and cell exposure to 3AC (a molecule that inhibits the enzymatic activity of SHIP). In both, SHIP-1 siRNA transfected (Figure 2A-C) or 3AC-exposed CLL cells (Figure 2D); we observed that BCR ligation was incapable of inducing an increase of p-AKT levels, suggesting that SHIP is responsible for the activation of AKT upon BCR ligation in CLL cells. Since AKT could not be activated by BCR ligation in these cells, the involvement of SHIP in the reduction of AKT activation after BCR-Fc γ RIIb coligation could not be assessed. In contrast, in both, SHIP-1 siRNA transfected or 3AC-exposed CLL cells, activation of ERK (successfully achieved upon BCR ligation), could not be inhibited by BCR-Fc γ RIIb coligation, demonstrating that SHIP is involved in the inhibitory effect of Fc γ RIIb through inhibition of ERK signaling in CLL cells. In normal B cells, the ability of Fc γ RIIb-BCR coligation to inhibit p-ERK was not altered by 3AC exposure or SHIP-1 siRNA transfection (*Online Supplementary Figure S3*).

We further explored whether BCR-Fc γ RIIb coligation could inhibit the BCR-induced activation of CLL cells. Similarly to normal B cells (*Online Supplementary Figure S4A-B*), the crosslinking of Fc γ RIIb and BCR with whole anti-IgM pAb significantly inhibited the increase in Ca²⁺

flux (Figure 3A) and the increase in the proportion of CD69⁺ cells (Figure 3B) induced by BCR. We also observed that Fc γ RIIb-BCR coligation reduced the percentage of apoptotic cells induced by BCR activation after 48 hours (h) with or without IL4/CD40L, with no inhibitory effects being observed in proliferation ratios after 72h (Figure 3C-D, *Online Supplementary Figure S5*). In normal B cells, the increase of Ca²⁺ flux, CD69⁺ cells and proliferation induced by BCR ligation was reduced by Fc γ RIIb-BCR coligation, but no changes were detected in apoptosis (*Online Supplementary Figure S4*).

Collectively, our study shows that Fc γ RIIb-BCR coligation can reduce BCR signaling in CLL cells responsive to BCR by reducing the downstream activation of AKT and ERK pathways. Moreover, our results indicate that SHIP may be involved in the Fc γ RIIb inhibitory effect by blocking the ERK pathway. This inhibitory effect compromises BCR-induced leukemic cell activation, since molecular and cellular inhibitory effects were reverted when coligation was abrogating by using a specific anti-human Fc γ RIIb monoclonal antibody. These data are in agreement with a recent report published by Lemm *et al.*¹² which suggests that SHIP is a potential regulator of BCR signaling in CLL cells and may explain our previous findings suggesting a correlation between high expression of Fc γ RIIb and better prognosis in early stage CLL.¹⁵

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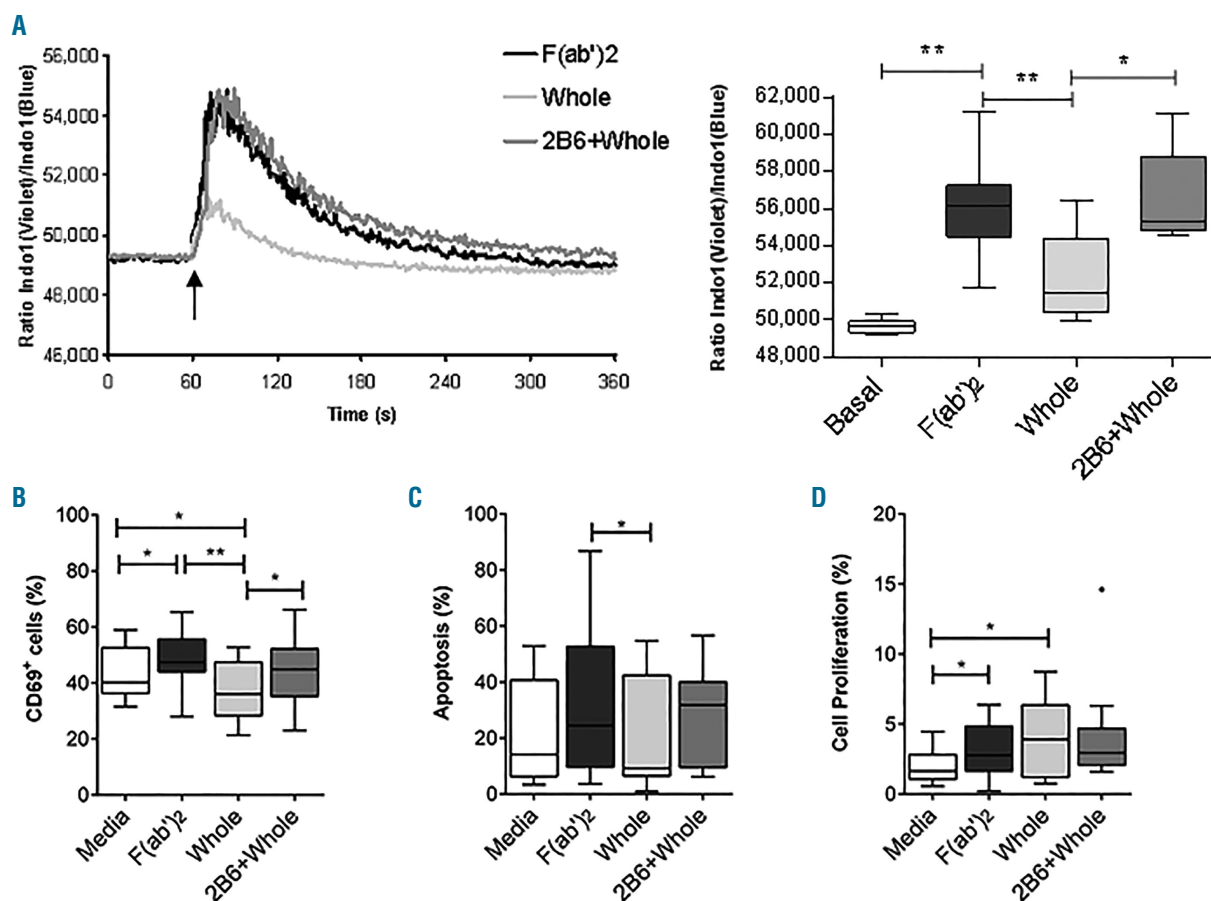


Figure 3. Activation, apoptosis and proliferation of chronic lymphocytic leukemia cells upon B-cell receptor (BCR) ligation or Fc γ RIIb-BCR coligation. (A) Calcium kinetics for a representative chronic lymphocytic leukemia (CLL) patient's sample in which purified leukemic cells were loaded with Indo1 ratio and acquired for 60 seconds prior to the addition of F(ab'), anti-immunoglobulin M (anti-IgM), whole anti-IgM or anti-Fc γ RIIb. (B) Box plots represents the median mean fluorescence intensity ratio of Indo1 (4 μ M) for each stimuli in CLL patient's samples (n=10). Purified B cells from CLL patients with responsive BCR were exposed to the indicated stimuli for 48 hours (h) or 72h and then analyzed by flow cytometry for CD69 expression on viable cells (B), Annexin V and TO-PRO³-3 (Annexin V⁺ cells were considered apoptotic cells) (C) or EdU incorporation using the Click-iTTM EdU Alexa FluorTM 488 Flow Cytometry Assay Kit (D) (n=10). Box plots encompass the data points within 1.5-times the interquartile range from the first or third quartile. P-values were calculated using two tail paired t-test (* P <0.05, ** P <0.01). F: F(ab'), anti-human IgM polyclonal antibodies (pAb); W: whole anti-human IgM pAb; 2B6: specific anti-human Fc γ RIIb monoclonal antibody.

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