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FcγRIIb-BCR co-ligation inhibits BCR signaling in chronic lymphocytic leukemia.

Rosa Bosch #1,2,3, Alba Mora #1,2,3,4, Carolina Cuellar1,2,3, Gerardo Ferrer5, Sergey Gorlatov6, Josep Nomdedéu7, Emili Montserrat8, Jorge Sierra1,2,3, Kanti R. Rai5, Nicholas Chiorazzi5 and Carol Moreno1,2,3,4.

1Laboratory of Oncology/Hematology and Transplantation, Institute of Biomedical Research, IIB Sant Pau, Barcelona, Spain; 2Department of Hematology, Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Spain; 3Biomedical Research Institute (IIB-Sant Pau) and José Carreras Leukemia Research Institute; 4Department of Medicine, Autonomous University of Barcelona, Barcelona, Spain; 5Karches Center for Oncology Research, The Feinstein Institute for Medical Research, Manhasset, New York, United States; 6MacroGenics, Inc., Rockville, MD, United States; 7Laboratory of Hematology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; and 8Department of Hematology, Institute of Hematology and Oncology, Hospital Clínic, IDIBAPS, Barcelona, Spain. #RB and AM contributed equally to this job.

Corresponding author: Carol Moreno MD, PhD. Hematology Department Hospital de la Santa Creu i Sant Pau, Mas Casanovas, 90, 08041 Barcelona, Spain. Phone: 0034-935565647; Fax: 0034-935565601; e-mail address: cmorenoa@santpau.cat
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 co-ligated with the BCR by cognate immune complexes (IC) and contributes to maintain peripheral tolerance and to shape the B-cell repertoire. FcγRIIb-BCR co-ligation 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 IgM (10µg/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 (Supplementary Figure 1). BCR responsiveness was not significantly associated with FcγRIIb expression (p=0.053) nor with the IgM expression (p=0.307).

As a subset of CLL patients show high levels of circulating IC and express polyan-reactive BCRs (6), 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 co-ligation 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 R. et al. suggested that FcγRIIb may inhibit BCR signaling in CLL cells by reducing ERK phosphorylation (11). Here, similarly to what we observed in normal B cells (Supplementary Figure 2A-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 co-ligation blocked the BCR-induced increase of p-AKT and p-ERK levels (Figure 1C). These inhibitory effects of FcγRIIb were reverted when co-ligation with BCR was abrogated by adding the specific anti-FcγRIIb monoclonal antibody 2B6 to the whole anti-IgM pAbs. Interestingly, ligation of BCR alone also induced a significant increase of p-SHIP levels, as reported before (13, 14) indicating that SHIP can also function independently of FcγRIIb.

We also studied the potential involvement of SHIP in the inhibition of p-AKT and/or p-ERK induced by FcγRIIb-BCR co-crosslinking. For that purpose, we inhibited SHIP using two different approaches; cell transfection with a specific SHIP-1 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 not capable to induce 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-FcγRIIb co-ligation 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-FcγRIIb co-ligation, demonstrating that SHIP is involved in the inhibitory effect of FcγRIIb through inhibition of ERK signaling in CLL cells. In normal B-cells, the ability of FcγRIIb-BCR co-ligation to inhibit p-ERK was not altered by 3AC exposure or SHIP siRNA transfection (Supplementary Figure 3).

We further explored whether BCR-FcγRIIb co-ligation could inhibit the BCR-induced activation of CLL cells. Similarly to normal B-cells (Supplementary Figure 4 A, B), the crosslinking of FcγRIIb and BCR with whole anti-IgM pAbs significantly inhibited the increase in Ca^{++} flux (Figure 3A) and the increase in the proportion of CD69^{+} cells (Figure 3B) induced by BCR. We also observed that FcγRIIb-BCR co-ligation reduced the percentage of apoptotic cells induced by BCR activation after 48h with or without IL4/CD40L, with no inhibitory effects being observed in proliferation ratios after 72h (Figure 3C-D and Supplementary Figure 5). In normal B-cells, the increase of Ca^{++} flux, CD69^{+} cells and proliferation induced by BCR ligation was reduced by FcγRIIb-BCR co-ligation, but no changes were detected in apoptosis (Supplementary Figure 4).

Collectively, our study shows that FcγRIIb-BCR co-ligation 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 FcγRIIb 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 co-ligation were abrogating by using a specific anti-human FcγRIIb mAb. These data are in agreement with a recent report published by Lemm EA. Et al. which suggests that SHIP is a potential regulator of BCR signaling.
in CLL cells (12) and may explain our previous findings suggesting a correlation between high expression of FcγRIIb and better prognosis in early stage CLL (15).
References

Figure 1. Molecular effects of BCR ligation or FcγRIIb-BCR co-ligation in CLL cells with responsive BCRs. (A) Immunoblot for 2 representative CLL patient’s samples that responded to BCR ligation. Purified B-cells from CLL patients 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 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 2 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 BCRs (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 pAbs; W: whole anti-human IgM pAbs; 2B6: specific anti-human FcγRIIb monoclonal antibody.

Figure 2. Involvement of SHIP in the inhibitory effect of FcγRIIb-BCR colligation on CLL cells. (A) Relative SHIP expression in CLL B-cells transfected with 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 µM of 3AC or
EtOH. 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. 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-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.

Figure 3. Activation, apoptosis and proliferation of CLL cells upon BCR ligation or FcγRIIb-BCR co-ligation. (A) Calcium kinetics for a representative 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’)2 anti-IgM, whole anti-IgM or anti-FcγRIIb. (B) Box plots represents the median mean fluorescence intensity ratio of Indo1 (4µM) 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 48h or 72h and then analyzed by flow cytometry for CD69 expression on viable cells (B), Annexin V and TO-PRO®-3 (Annexin V+ cells were considered apoptotic cells) (C) or EdU incorporation using the Click-iTTM EdU Alexa Fluor™ 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’)2 anti-human IgM pAbs; W: whole anti-human IgM pAbs; 2B6: specific anti-human FcγRIIb monoclonal antibody.
Figure 2

A

B

Relative SHIP expression

Relative Ser473 AKT phosphorylation

Relative Thr202/Tyr204 ERK phosphorylation

Non-Transfected cells

Transfected cells

C

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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 healthy 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™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit.
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.
Supplementary Figure 4.
Supplementary Figure 5.