Upregulation of AXL and β-catenin in chronic lymphocytic leukemia cells cultured with bone marrow stroma cells is associated with enhanced drug resistance

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Despite the advent of even more effective therapies, Chronic Lymphocytic Leukemia (CLL) is still incurable and patients often develop drug resistance. We and others have found that bone marrow stromal cells (BMSCs) are excellent models for assessing the mechanism(s) by which stroma cells nurture CLL B-cells and we have shown that BMSC protects leukemic B-cells from spontaneous and drug-induced apoptosis. The leukemic B-cell derives survival signals from stromal cells and the bone marrow site is able to harbor residual leukemic B-cells protected from chemotherapy. Prior evidence indicates that the facilitation of residual disease burden may be a key pathway to clonal evolution and ultimate clinical relapses difficult to treat in CLL.

To further delineate additional resistance mechanisms, we evaluated alteration of critical survival pathways including AXL, in leukemic B-cells upon co-culture with BMSCs. All experimental details are provided in the supplement. We co-cultured primary CLL B-cells (Supplementary Table 1) with BMSCs derived from healthy subjects or untreated CLL patients (Supplementary Table 2) and compared them to CLL B-cells cultured alone for 48 h. CLL B-cells were separated from BMSCs and analyzed by western blot (WB) analysis. A significant increase of AXL expression in post co-cultured CLL B-cells were detected compared to CLL B-cells cultured alone (Fig. 1A). Further analysis also found an increased accumulation of β-catenin in post co-cultured leukemic B-cells from basal levels (Fig. 1A). However, we could not detect any significant alteration of cell surface AXL levels on these leukemic B-cells by flow analysis (Fig. 1B, C), indicating an increase in AXL expression restricted to the cytoplasm. Additionally, we tested other malignant cell types and observed a significant increase of both AXL and β-catenin in B lymphoma cell lines (Mino, Raji, and SU-DHL4) upon their co-culture with BMSCs (Fig. 1D). Furthermore, by RT-PCR using specific sets of primers we observed a significant increase of AXL mRNA levels but not β-catenin mRNA levels in post co-cultured CLL B-cells compared to that in CLL B-cells cultured alone (Fig. 1E, F). Next, we examined the expression of AXL and β-catenin in CLL B-cells after their exposure to BMSCs for 48 h using either a direct co-culture method versus co-culturing CLL B-cells with BMSCs separated via transwells. Interestingly, we found increased expression of both AXL and β-catenin in CLL B-cells only when CLL B-cells were in direct contact with BMSCs but not when separated by transwells (Fig. 1G).

To explore if CLL B-cell/BMSC interaction induces activation of AKT and ERK-1/2 MAPK, post co-cultured leukemic B-cells were analyzed for P-AKT and P-ERK-42/44 by WB. We detected significant increases in P-ERK-42/44 but not in P-AKT(S473) levels in post co-cultured CLL B-cells compared to CLL B-cells cultured alone (Fig. 1H). Our further analysis to define AXL activation status, revealed no change in P-AXL (Y702), one of the critical activation sites within the kinase domain of AXL in co-cultured CLL B-cells as compared to CLL B-cells cultured alone (Fig. 1I) despite significant overexpression of total AXL (Fig. 1I). Further total P-AXL levels were determined...
Fig. 1 (See legend on next page.)
by immunoprecipitation experiments. Consistent with our findings in Fig. 1J, we also could not detect any significant alteration in total P-AXL level in pre- or post-co-cultured CLL B-cells (Fig. 1I). Therefore, the function of increased AXL levels in co-cultured CLL-B-cells is likely independent of AXL tyrosine kinase activity and is a subject of our future studies.

Upregulation of AXL and β-catenin is associated with the induction of resistance to multiple chemotherapeutic agents in human cancer cells. To see if drug exposure caused further increases in AXL and β-catenin, we treated CLL B-cells with chemotherapy drugs (fludarabine, chlorambucil) used for CLL, at sub-lethal doses (determined from the dose-response curve; Supplementary Fig. 1A) in the absence or presence of BMSCs. After 48 h of fludarabine treatment, significant upregulation of AXL and β-catenin levels were discernible in CLL B-cells in presence of BMSCs as compared to untreated CLL B-cells with or without co-culture with BMSCs (Fig. 1K). In addition, the anti-apoptotic Mcl-1 protein expression level was also partially induced in the presence of BMSCs. Similar observations were also noted when CLL B-cells were treated with a sub-lethal dose (Supplementary Fig. 1B) of chlorambucil for 48 h compared to CLL B-cells without drug treatment in presence of BMSCs (Fig. 1L). Furthermore, treatment of co-cultured CLL B-cells with a sub-lethal dose (Supplementary Fig. 1C, D) of novel agent drugs that included ibritinib, AXL inhibitor (TP-0903), or venetoclax also showed upregulation of AXL and β-catenin, over that seen with BMSCs alone (Fig. 1M–O). Thus in vitro drug exposure facilitates further increases in both AXL and β-catenin for co-cultured CLL-B-cells consistent with cellular drug resistance.

Since it is known activated ERK and stabilized β-catenin translocate to the nucleus resulting in transcriptional activation of their target genes, we subjected CLL B-cells to cytoplasmic/nuclear fractionation following co-culture. Indeed we found increased levels of both active (non-phosphorylated) β-catenin (Ser33/37/Thr41) and P-ERK-42/44 in the nuclear fractions of CLL B-cells co-cultured with BMSCs compared to CLL B-cells cultured alone (Fig. 2A), however, increased AXL expression was only in cytosolic fractions (Fig. 2B). Activated ERK can inactivate GSK-3β via phosphorylation resulting in the accumulation of β-catenin. Here we found significant increases of P-GSK-3β(Ser9) and increases in P-ERK-42/44 and β-catenin expression (Fig. 2C) in co-cultured CLL B-cells. CLL B-cells were also treated with the ERK upstream MEK inhibitor PD98059, in the presence or absence of BMSCs. After 48 h, we found decreases in P-ERK-42/44 level and accompanying decreases in both AXL and β-catenin for CLL B-cells in the presence BMSCs (Fig. 2D). One study reported a positive correlation between c-Jun and AXL expression levels in head and neck squamous cell carcinoma patients. We indeed found increases of P-c-Jun(S73) protein albeit at variable levels in CLL B-cells co-cultured with BMSCs (Fig. 2E). We also detected increased levels of P-c-Jun(S73) in Mino, Raji, and SU-DHL4 cells co-cultured with BMSCs versus these cells cultured alone (Fig. 2F). Additionally, treatment of CLL B-cells in co-culture with the c-Jun upstream, JNK inhibitor SP600125, reduced AXL level, and variably β-catenin expression (Fig. 2G). Moreover, there is evidence that AXL can modify β-catenin levels, so we analyzed whether AXL is upstream of β-catenin in Mino cells. We co-cultured Mino cells with BMSCs after
Fig. 2 (See legend on next page.)
being transduced with a lentivirus expressing Cas9 and guide RNAs targeting AXL [as efficient transfection and CRISPR experiment were not feasible in primary CLL B-cells]. CRISPR-mediated reduction in AXL expression in primary CLL B-cells was not as effective in suppressing drug-induced killing of CLL B-cells in co-culture. CLL B-cells treated with the following drugs; fludarabine (FLU) (3.5 μM) or chlorambucil (CIT) (15 μM) or venetoclax (Ven) (2.5 mM) (K) or TP-0903 (TP) (0.15 μM) (L) alone or in combination with PD98059 (PD) (70 μM) for 48 h in co-culture with BMSCs and induction of cell death was assessed using flow cytometry after staining with annexin/propidium iodide. Each drug treatment was done with CLL B-cells from two patients in triplicate. Results are presented as mean values with SD. CLL patients (P1, P4, P5, P14, P19, P20, P30, P31, P52, P53, P62), normal BMSC (N1, N5–N8, N16, N17, N20, N23, N32, N33, N37, N49), and CLL BMSCs (P1, P2, P5, P7, P9, P11, P12) are indicated by arbitrary numbers. M AXL, β-catenin, P-ERK-42/44, and P-c-Jun expression levels in B-cells and BMSCs from CLL patients. Expression levels were determined on purified leukemic B-cell lysates obtained from CLL patients (n = 5) before treatment and while being treated or after treatment. Actin was used as a loading control. CIT indicates chemo-immunotherapy. CLL patients (P1–P5) are indicated by arbitrary numbers. ‘N’ represents a normal (healthy) control and ‘P’ represents a given patient. N Simplified model of CLL B-cell and BMSC interaction. BMSC induces expression of both AXL and β-catenin in CLL B-cells when co-cultured with BMSCs (blue arrow). Further increase in AXL and β-catenin expression is observed when drugs are added to the CLL B-cell and BMSC co-culture (double red arrows). Elevated level of P-ERK-44/2 in CLL B-cells in the presence of BMSCs, enhances AXL expression and β-catenin accumulation through GSK3β inactivation. Activation of c-Jun in presence of BMSCs also increases AXL expression level and may modulate the increase in β-catenin level (red broken arrow) in CLL B-cells. AXL also may regulate the β-catenin expression (red broken arrow) in CLL B-cells.
persistence of disease even in the era of ever more effective therapeutic approaches.

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Conflict of interest
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