Protein kinase C-β-dependent changes in the glucose metabolism of bone marrow stromal cells of chronic lymphocytic leukemia

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Abstract
Survival of chronic lymphocytic leukemia (CLL) cells critically depends on the support of an adapted and therefore appropriate tumor microenvironment. Increasing evidence suggests that B-cell receptor-associated kinases such as protein kinase C-β (PKCβ) or Lyn kinase are essential for the formation of a microenvironment supporting leukemic growth. Here, we describe the impact of PKCβ on the glucose metabolism in bone marrow stromal cells (BMSC) upon CLL contact. BMSC get activated by CLL contact expressing stromal PKCβ that diminishes mitochondrial stress and apoptosis in CLL cells by stimulating glucose uptake. In BMSC, the upregulation of PKCβ results in increased mitochondrial depolarization and leads to a metabolic switch toward oxidative phosphorylation. In addition, PKCβ-deficient BMSC regulates the expression of Hnf1 promoting stromal insulin signaling after CLL contact. Our data suggest that targeting PKCβ and the glucose metabolism of the leukemic niche could be a potential therapeutic strategy to overcome stroma-mediated drug resistance.

KEYWORDS
Bone marrow stromal cells, chronic lymphocytic leukemia, glucose metabolism, PKCβ
1  |  INTRODUCTION

Chronic lymphocytic leukemia (CLL) continues to be the most common leukemia in adults in the Western world, characterized by an accumulation of monoclonal mature B cells in patients’ peripheral blood, lymph nodes, and bone marrow. The treatment for patients with CLL is becoming more individualized. Protein kinase inhibitors like ibrutinib, idelalisib, and the BH3 mimetic venetoclax have further improved the therapeutic options for CLL. However, the side effects in a large cohort of patients are critical. Despite this treatment options, CLL remains an incurable disease. CLL cells escape cell death in vivo, but rapidly die ex vivo unless a number of extrinsic microenvironmental factors are provided. This bidirectional communication is a dynamic process that has been shown to provide protection from spontaneous apoptosis. To date, it is well established that the CLL pathogenesis is dependent on the presence of various cell types including T cells, monocytes, nurse-like cells, and stromal cells and different soluble factors. Moreover, the increased understanding of the critical role of the microenvironment in resistance of CLL cells toward drug-induced apoptosis, minimal residual disease, and finally relapses has fostered the development of alternative therapeutic approaches.

Protein kinase C (PKC) has been discovered to participate in various biological functions including cell proliferation, differentiation, apoptosis, and tissue development. In B cells, PKCβ is highly expressed and plays a central role in propagating the nuclear factor ‘kappa-light-chain-enhancer’ (NF-kB) signaling downstream of the B-cell receptor. Studies in Prkcb−/− (PKCβKO) mice revealed that PKCβ is essential for B-cell-fate decisions. PKCβKO mice are also insusceptible to CLL transplantation emphasizing the role of the PKCβ kinase in bone marrow stromal cells supporting CLL development. In addition, the finding that the Lyn kinase in the tumor microenvironment (TME) is relevant in CLL pathogenesis supports the hypothesis that B-cell receptor-associated kinases (BAKs), like PKCβ and Lyn, are essential to shape the communication between malignant B-cells and the TME.

Furthermore, PKCβ is known as a negative regulator in insulin signaling and is involved in glucose transport in adipocytes. It facilitates metabolic reprogramming and mitochondrial remodeling in activated B cells. In addition, the induction of PKCβ in the microenvironment upon CLL contact was neither species restricted, nor limited to a subtype of stromal cells suggesting that CLL cells can interact with stromal cells through highly conserved mechanisms, for example, metabolic alterations. These data implicate that PKCβ participates in metabolism in different subsets of stromal cells. In the present study, we sought to investigate the metabolic functions of PKCβ in BMSC after CLL contact and the consequences underlying this cell-cell communication. These effects could be exploited to therapeutically overcome stroma-mediated drug resistance.

2  |  EXPERIMENTAL PROCEDURES

2.1  |  Patient samples

After informed patients consent and in accordance with the Helsinki declaration, peripheral blood was obtained from patients with a diagnosis of CLL. Studies were approved by the Ethics Committee of the University of Erlangen-Nürnberg (number: 219_14B, addendum 59_17 Bc). Peripheral blood mononuclear cells (PBMC) isolation was performed as previously described.

2.2  |  Cell culture

PKCβKO and WT BMSC were established from femur and tibiae of 4- to 8-week-old Prkcb−/− and Prkcb+/+ mice accordingly. The culture conditions of CLL cells, EL08-1D2 cells, PKCβKO BMSC, and following cocultures were previously described in detail. Purified CD19+ CLL cells were cocultured on BMSC or EL08-1D2 cells for 5 days. CLL cells were physically removed from stromal cells by repeated washing resulting in minimal cross-contamination numbers. In flow cytometry analysis, CLL cells can easily be distinguished using a CD19+ lymphocyte gate. BMSC were further purified using anti-CD19 magnetic beads to eliminate any minimal CLL contamination especially in quantitative polymerase chain reaction (qPCR) analysis (Figure S1A).

2.3  |  Apoptosis assay

Apoptotic cells were assayed by Annexin-V staining. Cells were washed with phosphate-buffered saline (PBS) and resuspended in 500 mL Annexin binding buffer (BD Bioscience, San Jose, California) containing 5 μL of Annexin V-FITC (Biolegend, San Diego, California) and 10 μL of 50 μg/mL propidium iodide (PI, Sigma-Aldrich, St Louis, Missouri). Apoptotic cells were assayed by Annexin-V staining. Cells were washed with phosphate-buffered saline (PBS) and resuspended in 500 mL Annexin binding buffer (BD Bioscience, San Jose, California) containing 5 μL of Annexin V-FITC (Biolegend, San Diego, California) and 10 μL of 50 μg/mL propidium iodide (PI, Sigma-Aldrich, St Louis, Missouri).
Darmstadt, Germany) to determine the phosphatidylserine exposure on the outer plasma membrane. After incubation for 15 minutes at 4°C, the specimens were quantified by flow cytometry.

2.4 | DNA fragmentation assay

To identify fragmented DNA, a terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling assay (Tunel) was performed (Apo-Direct-Kit, BD Bioscience). CLL cells were fixed in 1% paraformaldehyde for 15 minutes on ice. Cells were washed twice in PBS and collected in 500 μL PBS and 1 mL 70% ice cold ethanol. After 30 minutes, CLL cells were washed twice in washing buffer and resuspended in staining solution containing fluorescein dUTP concerning manufacturer’s instructions. Fluorescein incorporated in DNA strand breaks was detected by flow cytometry.

2.5 | Mitochondrial membrane potential

To investigate the mitochondrial membrane potential ΔΨM, the cells were stained with 20 nM 3,3′-dihexyloxacarbocyanineiodide (DiOC6 (3), Thermo Fisher Scientific, Waltham, Massachusetts) for 30 minutes at 37°C. CLL cells were centrifuged and resuspended in 500 μL buffer (5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bovine serum albumin (BSA) in PBS), containing 5 μL of 20 μg/mL PI, and were analyzed after 10 minutes by flow cytometry.

2.6 | Glucose uptake

After coculture CLL and stromal cells were separated and cultured in medium without glucose for 4 hours. The fluorescent glucose analog 6-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (6-NBDG, Thermo Fisher Scientific, Waltham, Massachusetts) was added to the cells following the staining protocol recommended by the manufacturer (Glucose uptake cell-based assay kit, Cayman Chemicals, Ann Arbor, Michigan) and glucose uptake was semiquantified by flow cytometry.

2.7 | Adenosine triphosphate levels

After separating the coculture, the adenosine triphosphate (ATP) concentration of CLL and stromal cells was assessed using a colorimetric ATP Detection Assay Kit (Cayman Chemicals) according to the manufacturer's recommended protocol.

2.8 | RNA preparation and quantitative real-time PCR (qPCR)

RNA was extracted from cell lysates after coculture separation using anti-CD19 magnetic beads (Qiagen, Hilden, Germany) and cDNA was prepared using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Penzberg, Germany) as suggested by the manufacturer. cDNA was analyzed by quantitative real time qPCR (StepOnePlus System, Applied Biosystems, California) using the SYBR Select Master Mix (Life technologies, Carlsbad, California) according to the manufacturer's protocol. Relative gene expression was determined by normalizing target gene expression to β-actin using gene-specific and species-specific primers (Table S1). All primers were produced by Eurofins Genomics (Ebersberg, Germany).

2.9 | RT² assays

After coculturing, CLL and stromal cells were separated with anti-CD19 magnetic beads and RNA from stromal cells was extracted. Stromal cDNA was prepared from RNA (RT² First Strand Kit, Qiagen) and subjected to pathway-focused gene expression analyses of 84 genes involved in glucose metabolism (RT² Profiler PCR Arrays, Qiagen). The data were analyzed using the ΔΔCT method. The results after coculture with primary CLL cells from three individual patients were presented in scatter plots, heatmap, and a volcano plot.

2.10 | Flow cytometry (FACS)

Cells were stained according to the manufacturer's recommendations using fluorochrome-coupled antibodies or chemical dyes (Table S2). For intracellular staining, the cells were treated with Fix & Perm Cell Permeabilisation Kit (Life Technologies). Cells were analyzed using a FACSCanto flow cytometer (BD Bioscience) and the Kaluza 2.1 software (Beckman Coulter, Krefeld, Germany).

2.11 | Cellular and mitochondrial reactive oxygen species level

Total cellular reactive oxygen species (ROS) was quantified by FACS analysis using CellROX Deep Red reagent (Thermo Fisher Scientific). To discriminate early apoptotic states, cells were co-stained with Annexin-V. Mitochondrial ROS was analyzed using MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific). Fluorescence intensities were semiquantified by FACS analysis. Assessing the contribution of mitochondria to ROS, cocultures were incubated with the mitochondria-targeting antioxidant Mitoquinone (MitoQ, 500 nM) for 12 hours.

2.12 | Mitochondrial mass

Cocultures were separated after 5 days. Cells were stained with a mitochondrial specific dye (MitoTracker Green, Thermo Fisher Scientific) according the manufacturer's instructions and analyzed with FACS.
2.13 | Antioxidant capacity

Cells were harvested and homogenized in cold buffer (5 mM potassium phosphate, pH 7.4, 0.9% NaCl, 0.1% glucose). Cell lysates were stained concerning the manufacturer’s instructions (Antioxidant Assay Kit, Cayman Chemicals). The antioxidant capacity was measured by fluorescence using a plate reader (SpectraMax M3, Molecular Devices, San Jose, California).

2.14 | Insulin enzyme-linked immunosorbent assay

The concentration of insulin was assessed in supernatants from EL08-1D2 and PKCβKO BMSC monocultures or cocultures with CLL cells after 5 days using an enzyme-linked immunosorbent assay (Mouse insulin ELISA Kit, Thermo Scientific).

2.15 | Statistical analysis

All experiments were repeated at least three times. The sample sizes for each experiment are provided in the figure legends. Statistical analyses were performed by one-way ANOVA for multiple comparisons. Two-tailed Student t-tests with unpaired or paired analysis were performed based on the distribution levels using GraphPad Prism Version 8 (GraphPad Prism Software Inc., La Jolla, California). Throughout the manuscript, statistical significance was defined as ****P < .0001, ***P < .001, **P < .01, *P < .05 or statistically nonsignificant (ns).

3 | RESULTS

3.1 | Stromal PKCβ-dependent alterations on the glucose metabolism of CLL cells

We previously established a coculture system to study heterotypic cell-cell interactions between stromal and primary CLL cells.9 The EL08-1D2 cells are primary stromal cells derived from mouse embryonic (E11) liver supporting human hematopoietic stem cell activity.27 Therefore, EL08-1D2 stromal cells serve as control for stromal PKCβ induction which is required for stromal cell-mediated protection of CLL cells. To eliminate possible off-target effects of siRNA or shRNA manipulation in stromal cells, we recapitulate our experiments with primary BMSC from Prkcb−/− knockout mice. In addition, pivotal experiments were repeated with BMSC from Prkcb+/+ WT mice. A survival profile from EL08-1D2, PKCβKO, and WT BMSC during a time course from 1 to 5 days of coculture with and without CLL cells showed that the stromal cells have a comparable viability over the time (Figure S1B).

To assess to what extent PKCβ expression in mesenchymal stromal cells can influence the metabolism, we measured glucose uptake (Figure 1A) and density of the glucose transporters Glut3 and Glut4 (Figure 1B) in primary CLL cells cultured alone or cocultured with EL08-1D2 or PKCβKO stromal cells. After CLL cell separation, glucose uptake in living CLL cells (CP) was quantified by FACS based on the incorporated fluorescent glucose analog 2-NBDG-FITC (n = 4) and, density of the glucose transporters Glut3 and Glut4 was assessed in CLL cells after contact to EL08-1D2 stromal cells and, Glut3 and Glut4 was assessed in CLL cells after contact to EL08-1D2 stromal cells and PKCβKO BMSC (n = 5). The same patient-derived CLL cells were used for different Glut transporter stainings. Survival of CLL cells on EL08-1D2 and PKCβKO BMSC (left panel) and CLL monoculture (right panel) was measured under baseline conditions and upon glucose administration (20 mM) after 5 days (n = 3). E,F, The density of the glucose transporters Glut3 and Glut4 was assessed in CLL cells after contact to EL08-1D2 stromal cells and PKCβKO BMSC under high glucose conditions (n = 5). Bars indicate the SE of the mean. *P < .05; **P < .01; ****P < .0001.

BMSC, bone marrow stromal cells; CLL, chronic lymphocytic leukemia cells; CP, CLL cell pellet after stromal contact; FITC, fluorescein isothiocyanate; Glut, Glucose transporter; 2-NBDG, (2-[N-7-nitrobenz-2-oxa-1,3-diazol-4-yl] amino)-2-deoxy-d-glucose); p, P-value; PKCβ, protein kinase C-β.
consumption and the content of lactic acid in the supernatant of EL08-1D2/CLL and PKCβKO/CLL cocultures after 5 days. Production of lactic acid, a surrogate for aerobic glycolysis, in PKCβKO/CLL cocultures was clearly enhanced compared to EL08/CLL cocultures expressing stromal PKCβ (data not shown). In this study, we focused on the direct contact between CLL and stromal cells because there is no stromal PKCβ induction that is crucial for following CLL survival using transwell experiments to separate the different cell compartments (Figure S1C,D). Next, we detected the glucose uptake based using transwell experiments to separate the different cell compartments. Stromal cells proficient or depleted of PKCβ late apoptotic stages in primary CLL cells before and after coculture on EL08-1D2 stromal cells accompanied by an enhanced surface density of glucose transporter GLUT3 (Figure 1A,B), reflecting stromal-cell mediated metabolic studies in CLL cells from Jitschin et al. Glucose transporters GLUT1 and GLUT2 were not affected in these CLL cells (Figure S2A). Interestingly, the glucose uptake in CLL cells clearly increased further in cocultures with PKCβKO BMSC (Figure 1A) attendant by an increased GLUT4 expression in the CLL cells (Figure 1C). Surprisingly, high glucose conditions can rescue CLL survival on PKCbKO stromal cells, but the addition of glucose had no effect on the survival rate of CLL monocultures (Figure 1D). The expression of the Glut receptor GLUT4 raised in CLL cells due to high glucose medium conditions. Furthermore, we assessed a reduction of glucose transporter GLUT4 in CLL cells cocultured on PKCbKO BMSC under high glucose conditions. This effect is dependent on stromal PKCβ expression (Figure 1E,F).

### 3.2 Mitochondrial fitness in CLL cells

To detect the apoptotic mechanism of CLL cells, we analyzed early and late apoptotic stages in primary CLL cells before and after coculture on stromal cells proficient or depleted of PKCβ. We used an Annexin-V/PI staining to show the phosphatidylserine exposure in the early apoptosis. The late apoptotic stages were visualized via DNA strand breaks performing a Tunel assay. On EL08-1D2 cells, CLL cells had a clear survival benefit compared to PKCbKO/CLL cocultures. The reduction of Annexin-V-positive CLL cells on EL08-1D2 cells strongly correlated with a reduction of DNA strand brakcs within these cells. In contrast, stromal PKCβ depletion led to a higher number of Annexin-positive CLL cells in accordance to an increase of UTP-positive CLL cells (Figure 2A,B). This was also in line with the necrosis visualized by the increased PI signal (Figure 2C). This strong correlation between stromal PKCβ expression and CLL survival pointed to the importance of PKCβ for the antiapoptotic signals provided by EL08-1D2 cells. To deliver insight into the cellular stress level of CLL cells, we used a CellROX assay to detect and quantitate cellular ROS. Surprisingly, the intensity of ROS did not change in CLL cells cocultured on stromal cells compared to CLL monoculture (Figure 2D).

For assessing the mitochondrial fitness in CLL cells, we measured the mitochondrial-specific ROS production in CLL cells using a MitoSOX indicator staining. Interestingly, MitoSOX staining was in line with early and late apoptosis of CLL cells upon stromal contact. On EL08-1D2 stromal cells CLL cells survived and showed a diminished MitoSOX level. CLL cells cocultured on PKCbKO BMSC went into apoptosis and had a clearly enhanced MitoSOX expression (Figure 2E). Under the assumption that mitochondrial ROS might be a relevant CLL survival factor we treated the cocultures with the mitochondrial-targeted antioxidant mitoquinone (MitoQ) to suppress mitochondrial stress. MitoQ led to a reduction of MitoSOX in CLL cells on PKCβKO BMSC (Figure 2F). This protective effect of MitoQ strongly point to mitochondria as source of ROS overproduction in CLL cells cultured on PKCβKO BMSC.

In addition, we measured the cellular ATP production and the mitochondrial membrane potential (ΔΨm) as indicator for mitochondrial functionality. In fact, a subgroup of CLL cells, showing a low ATP level in monoculture, have a significantly higher ATP production in contact to PKCbKO BMSC (Figure 2G, right panel). These effects were not related to the viability of the different CLL cells in monoculture compared to coculture conditions (Figure 2G, lower panels). However, we detected a PKCβ-independent increase of ΔΨm in CLL cells cocultured on stromal cells (Figure 2H). PKCβ depletion in BMSC did not lead to mitochondrial depolarization in CLL cells since the mitochondrial mass in CLL cells did not change (Figure 2I). Of note, the PKCβ-dependent changes in the glucose uptake of CLL cells and their mitochondrial fitness could also be demonstrated in CLL cells cocultured with WT BMSC under the same conditions (Figure S3).

### 3.3 PKCβ expression lead to changes in glucose metabolism of activated BMSC

To understand the consequences of PKCβ expression in BMSC after CLL contact, we had a closer look on the metabolic changes in the stromal compartment. In following figures our results are focused on the BMSC cells.

We could show that stromal cells reduce the glucose uptake upon CLL contact in a PKCβ-independent manner (Figure 3A). In addition, we detected a PKCβ-dependent distribution of glucose transporter in BMSC after CLL contact. Glut1 and Glut4 were clearly reduced on EL08-1D2 stromal cells, expressing PKCβ after CLL contact, as opposed to EL08-1D2 monoculture. PKCβKO BMSC showed no significant changes in glucose transporter expression after CLL contact (Figure 3B). However, the PKCβ induction in stromal cells is not dependent on high glucose conditions (Figures 3C and S4B). Activation of stromal cells by direct contact to malignant B-cells induced PKCβ-dependent changes in the stromal gene expression of metabolic active enzymes (Figure 3D-F). We found changes in the expression levels of stromal genes, like aconitate-2 (Aco2), amyl-alpha-1, 6-glucosidase (Agl), isocitrate dehydrogenase-1 (Idh1), ATP citrate lyase (Acl), fumarate hydratase-1 (Fh1) glycoen synthase 2 (Gys2), and succinate-CoA ligase (SuccL2). These genes are associated with the tricarboxylic acid (TCA) cycle. Glycolytic enzymes including glucose-6-phosphate dehydrogenase (G6pd), glucose-6-phosphate isomerase (Gpi1), enolase-2 (Eno2), glycogen phosphorylase (Pgym), and hexokinase-2 (Hk2) were deregulated in a PKCβ-dependent manner indicating a shift towards oxidative phosphorylation in EL08-1D2 stromal cells (Figure 3E,F).
Mitochondrial fitness in CLL cells. A, Survival of CLL cells in medium (monoculture) and cultured on EL08-1D2 or PKCβ KO BMSC (CP) was assessed by Annexin-V/PI staining. Three representative histograms of a FACS analysis of CLL cells stained with Annexin-V/PI (right panel) were shown (n = 5). B, TUNEL assay was performed to detect late stages of apoptosis in CLL cells in absence or presence of EL08-1D2 or PKCβ KO BMSC after 5 days (n = 6). A representative histogram of UTP-FITC stained CLL cells is shown in the right panel. CLL in medium represented CLL cells in a monoculture (blue). CLL cells in direct contact to EL08-1D2 cells were dedicated in gray. CLL cells after contact to PKCβ KO BMSC were colored in red. C, Necrosis of CLL cells is shown separately by PI-positive CLL cells. The subdivision between Annexin-V+/PI low and Annexin-V+/PI high describe early apoptosis and late apoptosis/necrosis (n = 6). D, Intracellular ROS intensity in CLL cells before and after stromal contact is shown as CellROX (MFI) measured by FACS (n = 10). E, Mitochondrial-specific ROS production was measured in CLL cells stained with the MitoSOX probe and semiquantified based on the MFI by FACS without and with MitoQ treatment (n = 5). F, ATP levels were analyzed in lysates from CLL cells in presence or absence of BMSC for 5 days using a colorimetric assay (n = 6). CLL monocultures were differentiated by ATP high (left panel) and ATP low content (right panel). Cell viability of CLL monocultures was assessed with Annexin-V/PI staining at the time point of ATP measurement (lower panels). (H) The mitochondrial membrane potential ΔΨM was assessed in CLL cells before and after contact to BMSC using the 3,3’-dihexyloxacarbocyanineiodide (DiOC6(3)) dye by FACS (n = 5). A representative histogram of the ΔΨM in CLL cells is shown (right panel). (I) The mitochondrial mass was semiquantified in CLL cells before and after stromal contact based on the MFI of Mitotracker staining by FACS (n = 7). Bars indicate the SE of the mean. * P < 0.05; ** P < 0.01; **** P < 0.0001. ATP, adenosine triphosphate; BMSC, bone marrow stromal cells; CLL, chronic lymphocytic leukemia cells; CP, CLL pellet after stromal contact; FITC, fluorescein isothiocyanate; ΔΨM, mitochondrial membrane potential; MFI, median fluorescence index; PI, propidium iodide; p, P-value; PKCβ, protein kinase C-β; ROS, reactive oxygen species; UTP, uridintriphosphat
3.4 | PKCβ-dependent ROS regulation in BMSC

We can show that CLL contact leads to mitochondrial depolarization of BMSC. The ΔΨM of EL08-1D2 and PKCβKO BMSC clearly decreased after CLL contact accompanied by a significantly declined ATP production (Figure S5A,B). In addition, the mitochondrial ROS level in EL08-1D2 stromal cells upon CLL contact was upregulated in a PKCβ-dependent way (Figure S5C). Remarkably, the shrinking ΔΨM level was not correlated to early and late apoptosis in BMSC (Figure S5D,E). The stable apoptosis rate was conformed to the unchanged mitochondrial mass (Figure S5F).

CellROX intensity in the stromal cells because of CLL contact (Figure 4B). We hypothesized that stromal cells produce a higher amount of antioxidants to prevent apoptosis and to compensate the PKCβ-dependent ROS regulation. Using an antioxidant assay, we could measure that PKCβKO BMSC produce significantly more antioxidants due to CLL contact (Figure 4C).

3.5 | PKCβ deficiency promotes insulin signaling via stromal Hnf1

Next, we measured the expression of the insulin signaling regulator hepatocyte nuclear factor 1 (Hnf1) in stromal cells. Interestingly, Hnf1 is only enhanced in PKCβKO BMSC after CLL contact (Figure 4D). After adding recombinant insulin, Hnf1 is also increased in EL08-1D2 stromal cells upon CLL contact (Figure 4E).
FIGURE 4  PKCβ-dependent effects on ROS regulation and insulin signaling in BMSC after CLL contact. EL08-1D2 and PKCbKO stromal cells were cocultivated with CLL cells for 5 days. A. After separation the stromal cells (SP) were subjected to gene expression using qPCR analysis. Changes in the relative gene expression of stromal neutrophil cytosolic factor 1 (Ncf1) were determined by qPCR in EL08-1D2 or PKCbKO BMSCs before and after CLL contact (n = 4). B. Intracellular ROS intensity in EL08-1D2 or PKCbKO BMSCs monocultures and cocultures with CLL cells is shown as CellROX (MFI) by FACS analysis (n = 6). C. The antioxidant capacity was measured in lysates from EL08-1D2 and PKCbKO BMSC in absence or presence of CLL cells (n = 6) using a colorimetric assay. D. Changes in the relative gene expression of the stromal transcription factor hepatocyte nuclear factor 1 (Hnf1) were analyzed by qPCR in EL08-1D2 or PKCbKO BMSC before and after CLL contact (n = 5) under basal conditions and, E, after insulin treatment (200 nM) (n = 5). F. The relative gene expression of stromal nitric oxide synthase 2 (Nos2) in EL08-1D2 and PKCbKO BMSC monocultures and after contact to CLL cells was detected (n = 4). G. Concentration of stromal insulin was assessed in supernatants from EL08 and PKCbKO BMSC before and after CLL contact using a mouse specific insulin ELISA (n = 8). H. CLL survival was analyzed by Annexin-V/PI staining cocultured to EL08-1D2 or PKCβKO stromal cells under baseline conditions and insulin addition (n = 3). Bars indicate the SE of the mean. *P < .05; **P < .01; ***P < .001; ****P < .0001. BMSC, bone marrow stromal cells; CLL, chronic lymphocytic leukemia cells; MFI, median fluorescence index; p, P value; PKCβ, protein kinase C-β; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; SP, stromal cell pellet after CLL contact.
the inducible nitric oxide synthase 2 (Nos2) is known to promote insulin resistance.\textsuperscript{26-30} Therefore, we analyzed the Nos2 expression in EL08-1D2 and PKCbKO BMSC. The stromal expression of Nos2 is decreased in EL081-D2 stromal cells after CLL contact. Once, PKCβ is deleted in the stromal compartment, Nos2 expression increased significantly upon CLL contact (Figure 4F). To analyze if the activated insulin signaling via Hnf1 in PKCbKO BMSC after CLL contact lead to an effect in insulin secretion, we detected the stromal insulin level in the supernatant of EL08-1D2 and PKCbKO monocultures and cocultures using a mouse-specific insulin ELISA. CLL contact induced the secretion of insulin by EL08-1D2 stromal cells, which was PKCβ-dependent (Figure 4G). Remarkably, adding recombinant insulin to the cocultures resulted in increased CLL cell survival indicating that primary CLL cells were more protected from PKCβ-deficient stromal cells (Figure 4H). The PKCβ-dependent alterations in stromal glucose uptake, ROS regulation and insulin signaling could also be recapitulated in WT BMSC upon CLL contact (Figure S6).

4 | DISCUSSION

Here, we describe that the expression of stromal PKCβ due to CLL contact results in metabolic changes towards oxidative phosphorylation and altered ROS production in BMSC. In turn, stromal PKCβ is required to diminish mitochondrial stress and apoptosis in malignant B-cells by stimulating glucose uptake. Metabolic analysis on the interplay between stromal cells and CLL cells showed increased survival and modulation of the redox status of CLL cells.\textsuperscript{31} We can confirm studies that show an elevated glucose uptake and higher GLUT3 expression in CLL cells after stromal contact.\textsuperscript{11} We extended these studies to demonstrate that stromal PKCβ deficiency further increases the glucose uptake in CLL cells accompanied by an enhanced insulin-dependent glucose transporter GLUT4 (Figure 1). Interestingly, the CLL cells can be rescued on PKCβ-JKO stromal cells under high glucose conditions accompanied by reduced glucose transporter GLUT4. A similar effect was shown by Standaert et al, where the PKCβ deletion caused an increased glucose uptake in adipocytes.\textsuperscript{32} High oxidative stress levels are prominent features of CLL cells compared to normal B cells.\textsuperscript{33-35} Rising ROS makes CLL cells more dependent on cellular antioxidants to maintain a redox balance.\textsuperscript{31} However, we show that the high cellular stress level in CLL cells remains constant upon stromal contact. Notably, Annexin-negative CLL cells show a significantly higher CellROX intensity on stromal cells indicating that antia apoptotic CLL cells still express high cellular stress due to stromal microenvironment protection (Figures 2 and S7A). Recent studies found increased mitochondrial stress and elevated mitochondrial oxidative phosphorylation in circulating CLL cells in comparison to normal B cells.\textsuperscript{36} These studies identify the mitochondrial metabolism as key source for abundant ROS. Our results demonstrate that stromal contact changes the mitochondrial ROS in CLL cells. Direct contact to EL08-1D2 stromal cells clearly diminishes the MitoSOX expression in CLL cells, while stromal PKCβ deficiency leads to a further increase of the mitochondrial ROS level in CLL. In addition, CLL cells in direct contact to PKCβ-JKO BMSC showed a slightly reduced production of antioxidants, which might be a reason for elevated CLL cell death on PKCβ-JKO BMSC (Figure S7B). Thus, PKCβ expression in EL08-1D2 stromal cells resulted in decreased mitochondrial stress in CLL cells, which led to a maintenance of redox balance and promotion of cell survival. Surprisingly, stromal PKCβ deletion has no effect on mitochondrial depolarization and mitochondrial mass stability in CLL cells. In addition, the APT level of CLL cells seems not to change after stromal contact. More specifically, only CLL cells exhibiting low ATP levels show a significantly increased ATP amount after contact to PKCβ-deficient stromal cells, which could contribute to a chemoresistant phenotype.\textsuperscript{37}

Reports on metabolic activity in niche cells supporting survival of malignant B cells are limited to the effects in the tumor cells. Our data further documents that activated stromal cells show signs of mitochondrial depolarization accompanied by a clear loss of total ATP, but without any sign of apoptosis (Figure S5). The enhanced production of antioxidants in BMSC is essential to maintain the redox balance (Figure 4C), which is in line with data from Zhang et al.\textsuperscript{31} ROS, controlled by the NADPH oxidative complex, are an important inflammatory mediator. Our data demonstrate that stromal PKCβ leads to an increase of Ncf1. Ncf1 activates the NADPH oxidase and plays an essential role in immune response and autophagic flux.\textsuperscript{38,39}

One hallmark of tumor cells is the reprogramming of their metabolic pathways to cope with the elevated requirements of energy and macromolecules due to their high proliferation rate.\textsuperscript{40-42} We identified that stromal cells reduce the glucose uptake accompanied by diminished expression of glucose transporters Glut1 and Glut4, with Glut4 being the only insulin-responsive glucose transporter. Remarkably, several enzymes included in the TCA cycle are deregulated in PKCβ-JKO BMSC after CLL contact. Therefore, we assume that PKCβ-deficient BMSC have an increased glycolytic activity, which is in line with upregulated genes like Eno2 and Hk2 and the enhanced lactate production in PKCbKO/CLL cocultures. This implicates that PKCβ expression in EL08-1D2 stromal cells due to CLL contact activates the TCA cycle, the central hub of oxidative metabolism, which leads to a shift towards oxidative phosphorylation in these activated stromal cells. Oxidative phosphorylation yields more ATP using less glucose as compared to glycolysis. This excess of glucose might be an advantage for CLL survival upon stromal contact.

The hypothesis that upregulation of PKCβ in stromal cells might be related to metabolic alterations, is supported by data showing a potential role of PKCβ in regulating energy homeostasis and contributing to the development of metabolic syndrome.\textsuperscript{25} These studies pointed out that PKCβ is important for insulin resistance as well as for interacting with the pathogenesis of diabetic complications.\textsuperscript{43-46} The increased expression of the insulin signaling regulator Hnf1 in PKCβ-deficient BMSC upon CLL contact support PKCβ as a known negative regulator of the insulin signaling pathway. We hypothesize that as soon as PKCβ is expressed in EL08-1D2 stromal cells, Hnf1-mediated insulin
signaling could be blocked. Adding insulin to the cocultures led to a functional rising of Glut4 receptor expression in activated BMSC. Due to an inactive insulin signaling pathway in ELO8-1D2 stromal cells higher amounts of insulin were needed to assimilate glucose. This elevated insulin production was measured in the supernatants of ELO8-1D2 stromal cells in comparison to PKCβ-deficient BMSC after CLL contact. This was in line with the survival effect of recombinant insulin on CLL cells upon PKCβ KO BMSC contact.

5 | CONCLUSION

B-cell leukemia displays a natural niche residenzy in the bone marrow. Recent studys demonstrates the importance of PKCβ expression in the stromal compartment to protect malignant B-cells from cytotoxic therapies in vivo enrolling a PKCβ-dependent effect in environment-mediated drug resistance of CLL cells.7,48 Therefore, induced expression of PKCβ may be a common mechanism of malignant cells to harness stromal cells for tumor-promoting purposes. Here, we show that the expression of stromal PKCβ results in metabolic changes and altered ROS production in BMSC. Furthermore, PKCβ-deficient BMSC promotes stromal insulin signaling due to CLL contact. In addition, stromal PKCβ diminishes mitochondrial stress and apoptosis and promote glucose uptake in CLL cells. In summary, our study provides new insights in the PKCβ-dependent regulation and metabolic changes of the stromal niche cells upon CLL contact. Our data imply that targeting PKCβ and the glucose metabolism of the leukemic niche could be a potential therapeutic strategy in CLL.

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AUTHOR CONTRIBUTIONS

F.v.H.: collection and assembly of data, data analysis, and interpretation; M.F.: bioinformatics data analysis and interpretation; M.K.: administrative support in biostatistics; S.V.: provision of study material or patients, final approval of manuscript; A.N.K.: final approval of manuscript; R.A.J.O., M.L., A.E.: provision of study material; J.W.: provision of study material or patients; A.M.: administrative support, final approval of manuscript, and financial support; G.L.-G.: conception and design, financial support, data analysis, and interpretation and manuscript writing.

CONFLICTS OF INTEREST

The authors declared no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors declare in the paper and its supplementary information. All raw data are available from the corresponding author upon request.

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